MICROBIAL COMMERCIAL ACTIVITY NOTICE (MCAN)

TS0VSMC9

CONTAINED USE OF A GENETICALLY MODIFIED *SACCHAROMYCES CEREVISIAE* STRAIN FOR USE IN PRODUCTION OF ETHANOL

FILED BY

DANISCO US INC.
(Operating as DuPont Industrial Biosciences)

3490 Winton Place

ROCHESTER, NY 14623

Table of Contents

(a) Certificate Statement

Document Control Officer
TSCA Document Processing Center (7407)
Rm. L-100
Office of Pollution Prevention and Toxics
U.S. Environmental Protection Agency
401 M Street, S.W.
Washington, DC 20460

CERTIFICATION

I certify that to the best of my knowledge and belief the company named in this submission intends to manufacture, import, or process for a commercial purpose, other than in small quantities solely for research and development, the microorganism identified in this submission. All information provided in this submission is complete and truthful as of the date of this submission. I am submitting with this submission all test data in my possession or control and a description of all other data known to or reasonably ascertainable by me as required by 40 CFR §725.160.

The company identified in this notice has remitted the fee specified in 40 CFR §700.45(b).

Signature of authorized official Date

_____

Vincent J.H. Sewalt, Ph.D.
Senior Director of Product Stewardship & Regulatory
Danisco US, Inc.
(Operating as DuPont Industrial Biosciences)
925 Page Mill Road
Palo Alto, CA 94304

(b) Executive Summary

DuPont Industrial Biosciences (Danisco US, Inc.), formerly Genencor International, Inc., has constructed a *Saccharomyces cerevisiae* strain, and has determined that the microorganism is new under Section 5 of the Toxic Substances Control Act.

The genes expressed in *S. cerevisiae* are (see Appendix 1 and 3 for a detailed description of the strain construction):

The new strain, *S. cerevisiae* , will be used primarily in manufacture of fuel ethanol. It carries the expressed genes integrated within the genome as expression cassettes consisting only of yeast . No antibiotic resistance markers were inserted into the new microorganism.

A complete description of the production strain is provided in this submission.

The production of the *S. cerevisiae* strain is performed under Good Industrial Large Scale Practice (GILSP) containment, in closed fermentation systems using pure cultures. A description of the manufacturing steps including control technology is provided. The *S. cerevisiae* strain will be manufactured in existing manufacturing plants owned and operated by DuPont Industrial Biosciences. The yeast will be dried and the resulting active dried yeast (ADY) product will be supplied to customers' fuel ethanol plants, where the microorganisms contained in the preparation will be inactivated after the starch conversion to ethanol is completed.

(c) Confidential Business Claims

DuPont Industrial Biosciences are claiming certain strain construction, production process and volume information as confidential. Below are the answers to questions provided in 'Points to Consider in the Preparation of TSCA Biotechnology Submissions for Microorganisms,' pp. 52-53.

C. General questions:

1. For what period of time is a claim of confidentiality being asserted?

We request the strain information, production process and volume information be kept confidential for the indefinite future. Release of this information will give competitors knowledge about key components of this proprietary strain, the production process, cost structure and size of the facility. This information will give them unfair advantage on pricing and teach them DuPont Industrial Biosciences' proprietary production process.

We also request that the strain construction information be kept confidential for the indefinite future. As DuPont Industrial Biosciences has provided very detailed descriptions of not only the strain construction but also strain development strategies that will be employed on an ongoing basis, no finite time deadline can be identified at which CBI status will lapse.

2. Briefly describe any physical or procedural restrictions within the company or institution relating to use and storage of the information claimed as confidential.

The confidential information that relates to manufacturing operational procedures that are part of the company's internal operations documentation is being claimed as confidential. Procedures are written and noted for "internal" use only. There is restricted access to the plant facility limiting information exchange with outside persons. The confidential information that relates to strain construction is maintained as part of the intellectual property of the company.

All DuPont Industrial Biosciences employees sign confidentiality agreements. They are informed that this type of information is confidential. Disclosure beyond employees only occurs (1) to companies that are contractually bound to preserve confidential status, and (2) to government agencies under appropriate, narrow circumstances.

3. Has the information claimed as confidential been disclosed to others outside of the company or institution?

DuPont Industrial Biosciences process information is considered proprietary and is not disclosed to others outside the company. If vendors or others need to enter the production areas, it is encouraged to obtain a Non-Disclosure Agreement to safeguard against loss of confidential information. Production strain construction information is similarly considered confidential and is not disclosed to others outside the company.

4. Does the information claimed as confidential appear, or is it referred to, in any of the following:

(1) Advertising or promotional materials for the microorganism or the resulting end product

No. DuPont Industrial Biosciences is a technology company. Our intellectual property is critical to maintain and all employees must adhere to a strict internal review process with regard to disclosure of information that is or might be valuable.

(2) Material safety data sheets or other similar material for the microorganism or the resulting end product

No. Product MSDS disclosure is generally limited to exposure limits, clean-up recommendations and toxicity data. Formulation ingredients may be listed.

(3) Professional or trade publications

No. While DuPont Industrial Biosciences may publish some details of host strain characteristics, production strain construction is proprietary and not subject to publication.

(4) Any other media available to the public or to your competitors

No. Equipment vendors occasionally ask if we can be mentioned as a user of their equipment – that is normally approved at corporate level. Strain development techniques and strategies are not made public to the public or competitors.

(5) Patents

No. While some strain construction elements claimed to be confidential are the subject of patent publications, the production strain construction details of any particular strain are proprietary and not stipulated in patent publications held by DuPont Industrial Biosciences.

(6) Local, State or Federal Agency public files

No, to the best of our ability confidential information is not disclosed in these public files.

5. Has EPA, another Federal agency, a Federal court, or a State made any confidentiality determination regarding the information claimed as confidential?

No.

6. For each type of the information claimed as confidential, describe the harm to the company or institution's competitive position that would result if this information were disclosed.

There are several types of confidential information:
(1) Production quantities;
(2) Process descriptions and chemicals;
(3) Unit operation descriptions & process flow diagram;
(4) Possible organism release points;
(5) Possible worker exposure;
(6) Production strain Construction, including the identity of the newly introduced genetic elements.

(1) Dissemination of planned production volumes would indicate to competitors the scale of DuPont Industrial Biosciences' product introduction. Armed with this information, knowledgeable competitors could predict the target market(s) and allow them to build focused strategies to thwart the product introduction. Our company's future is highly dependent on successful introduction of valuable new technologies such as this.
(2) Dissemination of a process description would advance a competitor's process and product development capabilities in general, and potentially would also allow them to quickly develop a similar product.
(3) Dissemination of unit operations and particularly of a process flow diagram would also allow a competitor to analyze the process yield and determine the cost of manufacturing. This information would give a competitor an unfair competitive advantage against DuPont Industrial Biosciences. For example, a competitor armed with DuPont Industrial Biosciences' production cost information could price their product such that Danisco would be excluded from the market.

(4) Dissemination of possible organism release points tells a competitor what type of equipment is being used to contain and process the organism and the product. This is valuable information leading to pricing and/or process determinations.
(5) Dissemination of possible worker exposure as in #4 tells a competitor what type of equipment is being used, the number of persons involved in controlling the process and the duration of their potential exposure.
(6) Disclosure of the newly introduced genetic elements would teach a competitor which enzyme activities to introduce into the host organism to maximize biomass conversion efficiency; dissemination of strain construction techniques and strategies for developing a particular production organism tells a competitor which genes in the host organism to manipulate in order to maximize target enzyme production. In addition, such dissemination would teach competitors how to manipulate the noticed host gene sequences and inserted sequences in such ways that may overcome DuPont Industrial Biosciences' competitive advantage in the use of the host organism as a safe and suitable production organism for ethanol fermentations.

Making this information available to competition will allow them to analyze DuPont Industrial Biosciences' technical capabilities, production and cost structure and give them an unfair advantage in the market place.

7. If EPA disclosed to the public the information claimed as confidential, how difficult would it be for the competitor to enter the market for the resulting product?

Competitors have already entered this emerging market. If fully disclosed, competitors would have no trouble 'reverse-engineering' our product, process, or parts of our process, and this would allow them a competitive advantage in the specific target market for this product and in other markets. The new product is a very much enhanced version of an existing product with complementary side activities. Competitor introduction of a similar product would require specialized genetic technical expertise to construct the production microorganism and development of a specialized process to produce the intended end product. Competitors clearly have the expertise to do this type of work. Dissemination of our confidential information could very well lead to disastrous effects for Danisco in our target market.

(d) Submitter Identification

(1) Name
Danisco US, Inc.
(operating as DuPont Industrial Biosciences)

Headquarters address
3490 Winton Place
Rochester, NY 14623

(2) Name, address, and phone number for the principal technical contact
Vincent J.H. Sewalt
Senior Director of Product Stewardship & Regulatory

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(e) Microorganism Identity Information

(1) Description of the recipient microorganism and the new microorganism

(i) Data substantiating the taxonomy of the recipient and new microorganism to the strain level

The *S. cerevisiae* recipient strain used to create the new microorganism, [redacted], is a non-GM strain, which has been used widely in the commercial production of fuel ethanol in North America for several years [redacted] The species identity of the new microorganism has been confirmed through molecular sequencing.

The taxonomic identity of the donor strains is described under (e)(2)(i).

(ii) Information on the morphological and physiological features of the new microorganism

Saccharomyces cerevisiae cultures are typically fast growing at 28-33°C (Sherman 2002). At 37°C and above, growth rate is slower. Colonies appear white on media such as synthetic dextrose agar (SD) or richer media such as yeast extract, peptone and dextrose agar (YPD). Colonies are usually round. A characteristic sweet and alcoholic odor is produced by the culture. These features also apply to the new microorganism.

(iii) Other specific data by which the new microorganism may be uniquely identified for Inventory purposes

Features for unique identification for Inventory purposes are described above in (e)(1)(ii).

The new microorganism can be uniquely identified for Inventory purposes by using molecular biological methods such as PCR analysis using primers specific for the integrated genes. [redacted] An Identification Kit guiding identification of the production strain is available (see Appendix 4).

(2) Genetic construction of the new microorganism

(i) Data substantiating the taxonomy of the donor microorganisms

(ii) Description of the traits for which the new microorganism has been selected or developed and other traits known to have been added or modified

(iii) A detailed description of the genetic construction of the new microorganism, including the technique used to modify the microorganism; including a description of the introduced genetic material, including any regulatory sequences and structural genes and the products of those genes, how the genetic material is expected to affect behavior of the recipient; expression, alteration, and stability of the genetic material; methods for vector construction and introduction; and a description of the regulatory and structural genes that are components of the introduced genetic material, including genetic maps of the introduced sequences

In summary, the parent strain *S. cerevisiae* was transformed with

All these modifications were performed in such a way that no bacterial vector DNA remains present in the strain. No antibiotic resistance markers were inserted into the new microorganism. The final production strain was characterized by Southern blotting and PCR analyses.

A schematic overview of how the production strain was constructed is attached as Appendix 1; followed by a detailed description of the genetic construction of the new microorganism attached as Appendix 3.

(3) Phenotypic and ecological characteristics

(i) Habitat, geographical distribution, and source of the recipient microorganism

Saccharomyces cerevisiae is ubiquitous in nature. It has been recovered from a variety of sites under varying ecological conditions; mostly present on fruits, grains and other sources with a high concentration of carbohydrates, but is also common in soils. *S. cerevisiae* is not airborne, but needs a vector (e.g. an insect) to move within and between habitats. It is predominantly associated with environments favoring fermentation (preferring low to neutral pH) and is able to utilize several different carbohydrates depending on the type of metabolism involved (aerobic or anaerobic) as well as a wide variety of nitrogen sources.

(ii) Survival and dissemination under relevant environmental conditions including a method for detecting the new or recipient microorganism(s) in the environment and sensitivity limit of detection for these techniques

S. cerevisiae cultures are typically fast growing at 28-33°C (Sherman 2002). At 37°C and above, growth rate is slower. Colonies appear white on media such as synthetic dextrose agar (SD) or richer media such as yeast extract, peptone and dextrose agar (YPD. Colonies are usually round. A characteristic sweet and alcoholic odor is produced by the culture. These features also apply to the new microorganism.

The parent *S. cerevisiae* strain [ ] is a diploid and under right conditions can form spores. The spores can germinate and the resulting haploid cells could mate to form diploid strains again.

The new microorganism does not produce spores significantly during DuPont Industrial Biosciences' fermentation process. Spores that are present in the biomass waste are not resistant and are inactivated during biomass waste treatment before disposal.

It is not expected that the modifications will significantly affect the survival of the organism. This is supported by several survival studies on *S. cerevisiae* that have compared wild-type yeast and genetically modified yeast; they conclude that the genetically modified variants do not survive better than the wild-type (Broker 1990, Fujimura *et al.* 1994, Ando *et al.* 2005, Valero *et al.* 2005).

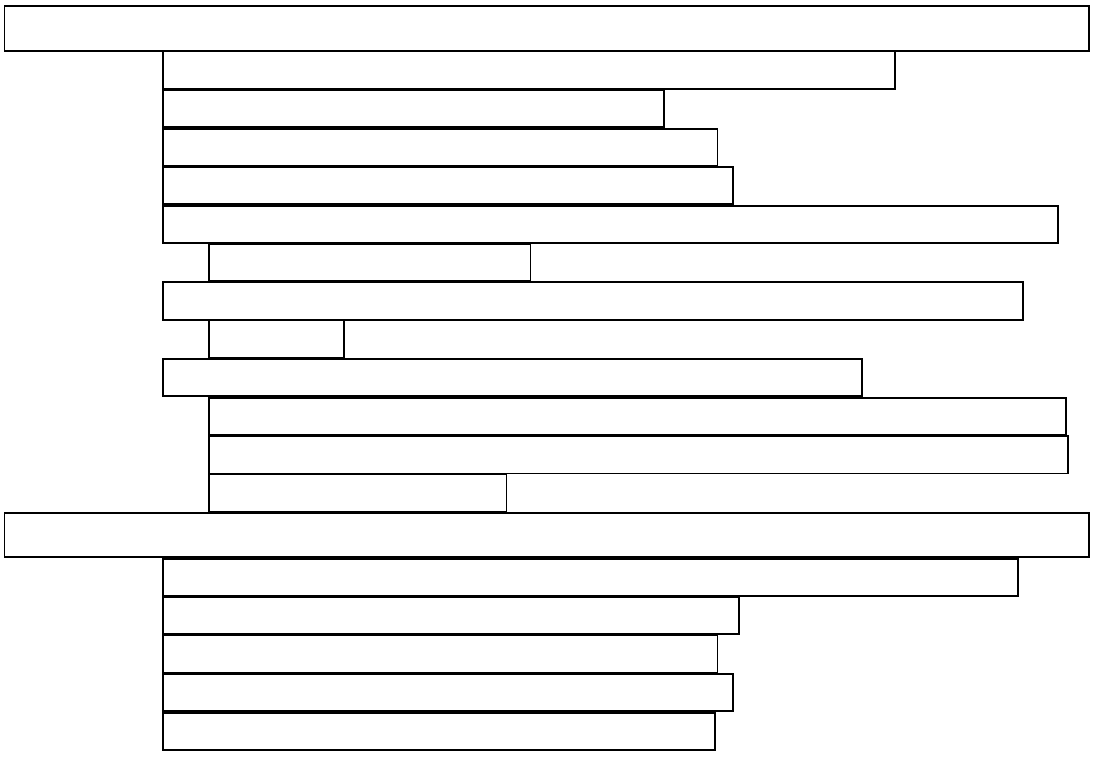
The new microorganism can be selected for using the features described under (e)(1)(ii) and (e)(1)(iii) above; including [redacted]

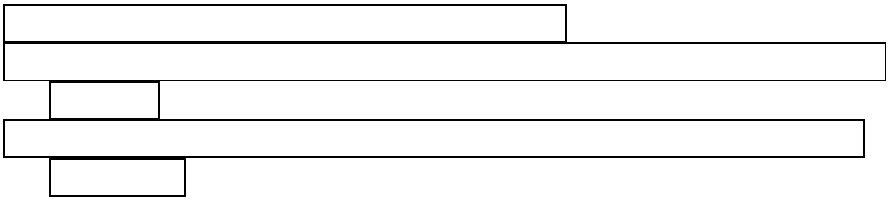
Once candidate production strain colonies have been isolated these can be identified using molecular biological methods like PCR analysis using primers specific for the integrated genes. An Identification Kit guiding identification of the production strain is available (see Appendix 4).

(iii) A description of anticipated biological interactions with and effects on target organisms and other organisms; a description of host range; a description of pathogenicity, infectivity, toxicity, virulence, or action as a vector of pathogens; and capacity for genetic transfer under laboratory and relevant environmental conditions

S. cerevisiae is a non-pathogenic fungus (see (j)(2)(i) below), and as such does not have a mammalian host nor does it have significant interactions with other organisms, other than saprophytic ones.

No vector bacterial DNA used in creation of the new microorganism remains in the new microorganism. Since no vector sequences are present in the final strain, the transfer frequency of the integrated expression cassettes is the same as for any other chromosomal sequence. [redacted]





(iv) A description of anticipated involvement in biogeochemical or biological cycling processes; involvement in rate limiting steps in mineral or nutrient cycling, or involvement in inorganic compounds cycling (such as possible sequestration or transformation of heavy metals)

S. cerevisiae is a facultative anaerobic saprophytic fungus. It is predominantly associated with environments favoring fermentation (preferring low to neutral pH) and is able to utilize several different carbohydrates depending on the type of metabolism involved (aerobic or anaerobic) as well as a wide variety of nitrogen sources. Although the new microorganism can [redacted] it is not expected to proliferate in the environment (see (e)(3)(ii) above).

(f) Byproducts – A description of byproducts resulting from the manufacture, processing, use, and disposal of the new microorganism

The primary *S. cerevisiae*-containing byproduct will be inactivated *S. cerevisiae* mixed with the byproducts of fermenting carbohydrate feedstock to ethanol, including feedstock components such as minerals, protein, fiber, oils, and non-digested sugars.

In addition to ethanol, *S. cerevisiae* [redacted] will produce volatile, semi-volatile and soluble organic metabolites in low to moderate concentrations, which are commonly associated with ethanol fermentation processes. Air and water emissions containing these volatile organic and water soluble compounds will meet applicable environmental permit limits.

No community exposure to viable *S. cerevisiae* [redacted] is expected, since the organism is inactivated (cell kill). In the yeast production and ADY manufacturing process, the cells are inactivated in the waste stream by using a combination of heat/temperature and pH. In the customer process (ethanol fermentation), the cells are inactivated during the ethanol distillation process and the distiller's grains drying process. During the distillation process, the cell matter is heated to greater than 80ºC and the distiller's grains drying process is in excess of 100ºC (providing adequate temperature for cell kill). Likewise, no community exposure to viable *S. cerevisiae* is anticipated from any of the byproducts or residuals generated in the downstream processes.

(g) Information on production volume

The new microorganism will be used in a fed-batch process to manufacture the desired ethanologen product. For each batch, the volume of the broth at harvest will be approximately [redacted]. At a cell density of [redacted] there will be approximately [redacted] and [redacted] wet cell mass produced per fermentor batch. Using bench scale experiments that model the large-scale production process, we have determined that [redacted] at the end of fermentation is equivalent to approximately [redacted] The cream is then [redacted] to form an Active Dry Yeast (ADY) product. The dry cell weight of the final product is [redacted]

[redacted] The table below approximates the cell mass to be produced in the next three years:

Production volume of new organism

Year	# of batches	DCW (kg) per batch	Total Dry Cells Mass Produced (kg)	Total Wet Cell Mass Fermenta-tion Broth (kg)	Total Wet Cell Mass Concen-trated Cream (kg)	Total ADY produced (kg)
1	[redacted]	[redacted]	[redacted]	[redacted]	[redacted]	[redacted]
2	[redacted]	[redacted]	[redacted]	[redacted]	[redacted]	[redacted]
3	[redacted]	[redacted]	[redacted]	[redacted]	[redacted]	[redacted]

(h) Use information

The new microorganism, *S. cerevisiae* strain [redacted], will be used to manufacture yeast ethanologen at DuPont Industrial Biosciences-controlled facilities and stored in approved controlled facilities. The product has application in industrial biofuel manufacture from biomass and grain. The ethanologen, [redacted] allows for efficient and more complete conversion of [redacted] into ethanol. The ethanologen will be inactivated during the ethanol distillation and removed from the fuel; and thus will only be present as inactivated cell mass in the residual distiller's grains (assuming grain feedstock) or biomass (assuming cellulosic biomass feedstock).

One such ethanol plant processing grain feedstock, [redacted] will use the improved ethanologen in their ethanol manufacture process (a process map is presented in Appendix 5).

The ADY ethanologen will be transported in vacuum sealed oxygen/air proof (Mylar) bags from the DuPont Industrial Biosciences facility (see section (i)(1)(ii) below) to the ethanol plant. The ethanologen in ADY product form will be stored on site in an appropriate storage area.

The ethanologen is added to a propagation tank where grain (corn) liquefact has been prepared using various materials such as accessory enzymes (e.g. glucoamylase, amylase, protease, phytase, etc.), a nitrogen source (e.g. urea), and specific salts/mineral to enhance yeast health. The ethanologen can be added directly to the tank or can be added to a mixing tank and transferred through process piping. The resulting hydrolysate containing all biomass and enzyme is then pumped in its entirety by a closed process to the large main ethanol fermentor.

The large main fermentor is prepared in a similar manner as the propagation tank by adding various materials to the liquefact such as accessory enzymes (e.g. glucoamylase, amylase, proteases, phytase, etc) and a nitrogen source (e.g. urea, ammonia). This prepared liquefact plus the entire contents of the propagation tank are then fermented for 40-60 hrs to process all the available starch. Upon completion of fermentation, this material is distilled to remove the ethanol from the aqueous components (water and solid mash components which includes the ethanologen). This aqueous process stream which includes the ethanologen is called stillage. This stillage is further processed to remove water and oil to create the target co-products of corn oil, syrup, WDGS (wet distiller grains with solubles), and DDGs (dried distillers grains with solubles).

The subject strain will be used for ethanol production on industrial scale; the current industry standard is between 730,000-807,000 gallons (2,800-3100 m^3). As the technology and economy of scale develops, this will likely continue to increase. . The current industry operating scale is between 50-100 million gallons ethanol per year, but larger plants are likely as the industry develops.

At the ethanol manufacturing plants, the potential for worker exposure to viable *S. cerevisiae* [redacted] will occur at the tank addition step similar to additions of current ethanologen.

The primary byproducts from the fermentation of grain (e.g. corn) to ethanol are carbon dioxide, corn oil, syrup and residual corn and biomass solids in a wet form called wet cake and a dry form called DDGs. The wet cake and DDGs will contain inactive *S. cerevisiae* biomass and inactivated enzyme proteins. The carbon dioxide as a result of fermentative metabolism is generally vented from the fermentation tanks as off-gas and in some plants recovered through compression. The corn oil is

separated via centrifugation from the aqueous process stream (stillage) during the evaporation process. The syrup is the final concentrated stillage from the evaporator.

(i) Worker exposure and environmental release

(1) For sites controlled by the submitter

(i) Identity of the site where the new microorganism will be manufactured

The stock culture and seed vial lot preparation of this new microorganism will be conducted at the DuPont Industrial Biosciences manufacturing facility in Rochester, New York. The address is:

Danisco US, Inc.
1700 Lexington Avenue
Rochester, NY 14606.

The sterile, sealed, cryo-protected vials will then be sent for the manufacture of the ethanologen to the DuPont Industrial Biosciences manufacturing facilities in Cedar Rapids, Iowa. The address is:

Danisco US, Inc.
1000 41st Avenue Drive, S. W.
Cedar Rapids, IA, 52404

(ii) Process description

The Cedar Rapids manufacturing plant is a secure facility operated 24 hours a day. People not employed by DuPont Industrial Biosciences entering the facility must check-in and be registered. The stock cultures are prepared in a controlled laboratory that is solely for that purpose; access to the lab is limited. The fermentor and processing equipment in Cedar Rapids is located in an area of the plant that is separate from office and laboratory space; access to the production areas is limited.

The fermentor vessels used in the DuPont Industrial Biosciences process for the production of *S. cerevisiae* are closed stainless steel tanks designed and built according to American Society of Mechanical Engineers (ASME) codes.

Piping connections such as fill line, draw line, sample port, and inoculation port are highly secure and . Positive steam pressure is maintained on the exterior side of each valve in contact with the fermentor to prevent contamination. Compressed air is filtered to remove airborne particles. All procedural steps and engineering standards described above are meant to insure that a pure culture fermentation is run.

After each fermentation step is completed and the contents of the fermentor have been transferred, the vessel is cleaned in place (CIP) with a (sterilization step) and the wash contents of the fermentor are directed to the equalization tank. Bench scale experiments have demonstrated that *S. cerevisiae* is not viable after . The cell will not be viable at Other production equipment is also cleaned with a in a similar manner.

The harvest broth is then . Prior to use, the equipment in this step is sanitized using various chemical and thermal techniques. The final cream is stored in cleaned/sanitized stainless steel tanks and/or totes and maintained under refrigerated conditions. When the process is completed, all equipment is cleaned in place (CIP) with a hot and the wash contents are directed to the equalization tank.

The cream is then either . The final ADY product is vacuum packaged into oxygen/air proof bags and sent to a refrigerated warehouse for storage and distribution to the customer. When the process is completed, all equipment (e.g. totes, vessels, filters, extruders, etc) is cleaned with hot and the wash contents are directed to the equalization tank where the contents are for inactivation of the ethanologen.

The major unit operation steps in the DuPont Industrial Biosciences process (Figure 1) are described in detail below.

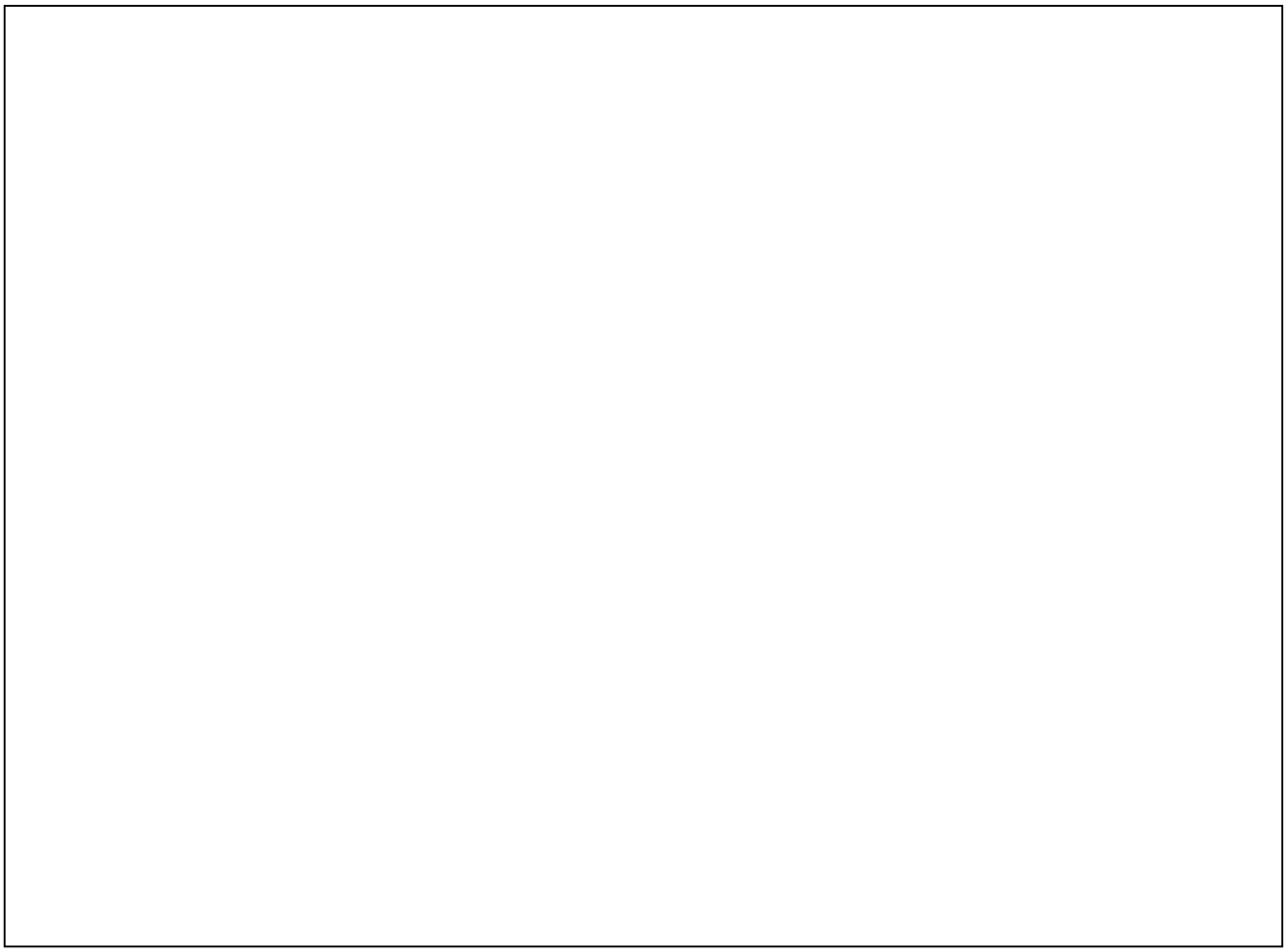


Figure 1. Process diagram for DuPont Industrial Biosciences manufacture of *S. cerevisiae*

a) Unit Operation Description

1) Stock culture preparation - The following procedures are performed in a bio-safety cabinet by laboratory staff equipped with the typical personal protective equipment (lab coat, safety glasses, latex or nitrile gloves). Starting with a frozen culture deposited in the Culture Collection, a flask of liquid medium is inoculated to grow the microorganism. After an appropriate population has grown up the culture is dispensed into cryogenic storage vials with 20% glycerol and stored under liquid nitrogen. Post storage quality assurance checks include 1) plating to check for the absence of contaminating organisms, and for cell survival and purity, 2) comparison of pre-freeze viability with the post-freeze viability to determine the survival rate and 3) testing the vials for productivity.

The seed vial lots are prepared in the Culture Collection laboratory according to a Standard Operating Procedure.

2) Aseptic inoculation of production organism - The seed inoculum is prepared by adding, in a bio-safety cabinet, vials of a stock culture cell suspension of the new microorganism (stored under liquid nitrogen) to a culture bag containing appropriate media. The bag is then placed in an incubator. When an appropriate cell mass is achieved, the contents of the bag is aseptically transferred directly into the seed tank by a member of the dedicated laboratory staff, equipped with lab coat, latex or nitrile gloves, and safety glasses.

3) Seed Fermentor - A seed fermentation vessel, controlled at prescribed environmental conditions (temperature, pH control via ammonia addition, pressure, and airflow), is used to generate the inoculum for the production vessel. Transfer to the production fermentor is conducted by dedicated fermentation operators and occurs via steam-sterilized hard piping.

After the fermentation is complete, the CIP process described in (i)(1)(ii) is carried out.

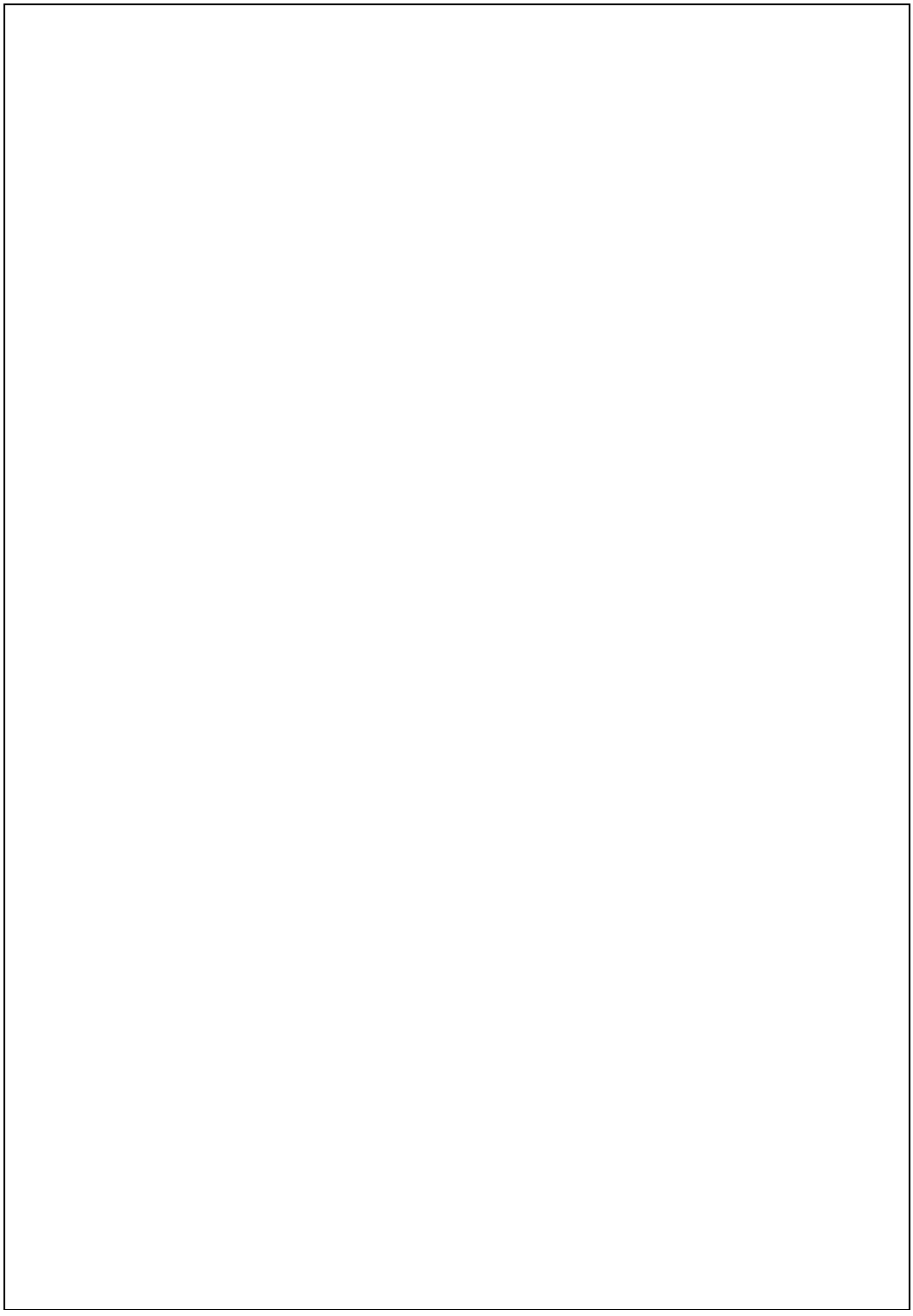
4) Main Fermentor - The process will use a defined medium as the basis for both seed and production fermentations.

The organisms transferred from the seed fermentor are allowed to grow until the target population size has been reached. The fermentor broth is then transferred to a drop tank.

Criteria are in place for detecting contaminated fermentations. If a run is deemed contaminated, it will not be used.
The fermentors used to grow the production organism are located in a separate area of the manufacturing facility and share an isolated holding tank system. After every run, the main fermentor is washed using a CIP system as described above in (i)(1)(ii). Rinse material from routine tank cleaning is directed to the equalization tank (where the pH is brought to neutral), which flows to the municipal waste treatment facility.

5) Post Fermentation Processing & Storage - The harvested fermentor broth from the main fermentor is sent to a drop tank The broth is then processed through a series of steps (see below) in order to transform it into ADY. After every batch, the fermentor and drop tank units are run through a CIP wash

as described above in (i)(1)(ii).



b) Starting Materials and Feedstocks

The *S. cerevisiae* [ ] fermentation is a contained process of a pure culture of the subject microorganism. The ingredients for the seed and main fermentors are as follows:

[ ]

c) Description of possible release points (see Figure 1)

1) Stock culture preparation - All manipulations are carried out in a bio-safety cabinet to maintain sterility and to protect the laboratory technician and the environment.
All disposable items (gloves, pipettes, culture preparation plates, culture spreaders) are placed in a biohazard trashcan, where they are then autoclaved. All glassware (culture flasks, beakers) is treated with bleach before being washed.
Consequently, there is no microorganism release expected at this stage.

2) Seed inoculum transfer - Once the cell mass in the culture flasks is sufficient, the culture is transferred into a stainless steel transfer apparatus in a bio-safety cabinet. Empty flasks are treated with bleach and rinsed to the sink (which ends up in the equalization tank before being released to the municipal waste water treatment system). Dedicated laboratory staff, equipped with lab coats, hard hats, gloves and safety glasses, transfer the inoculum from the transfer apparatus to the seed fermentor via steam sterilized hard piping. The empty transfer apparatus is caustic treated before being rinsed to the laboratory sink. Thus, there are no anticipated releases of the new microorganism at this stage.

3) Fermentation - The fermentors used to grow the production organism are located in a separate area of the manufacturing facility and share an isolated holding tank system. Tanks are routinely run through a [ ] (CIP) after every run as described in (i)(1)(ii). Rinse material from routine tank cleaning is directed to the equalization tank (where the pH is brought to neutral) that supplies the municipal waste treatment facility.

3a) Fermentation samples - Routine samples collected from the fermentor are either spot checked on the tank farm floor (ex., pH determination) or sent to the lab (on site) for routine analysis. All samples are inactivated with either chemical disinfectants or through autoclaving prior to disposal.

3b) Fermentor off gas - Fermenter exhaust gas passes [redacted] that reduces the amount of organism released to the environment. The exhaust air is directed [redacted]

4) Post fermentation processing & Storage – [redacted]

[redacted]

(iii) Worker Exposure

1) Stock culture preparation - Exposure to the organism is not expected during stock culture preparation due to the fact that transfers and organism handling occur in a bio-safety cabinet to maintain sterility and to protect the laboratory technician and the environment. Employee exposure to the subject microorganism is also limited at this step by the personal protective equipment worn by the laboratory technician. The stock culture preparation will require approximately [redacted] of lab technician time per stock culture.

2) Seed inoculum transfer - Exposure to the organism is not expected during seed inoculum transfer due to the fact that transfers use aseptic technique and are conducted in a bio-safety cabinet in a manner that contains the production microorganism. Employee exposure to the subject microorganism is also limited

at this step by the personal protective equipment worn by the laboratory technician.

After transfer of the inoculum to the seed fermentor, the empty transfer apparatus is given a treatment before being rinsed to the sump in the fermentor floor. When the apparatus is broken down for cleaning, it contains only inactivated organisms and residue.

3) Fermentation - The activities on the fermentation tank floor that involve potential worker exposure only regards the following: Process technicians, equipped with uniforms, latex or nitrile gloves, and safety glasses, will occasionally take samples from steam locked sample ports in the fermentor during production runs. The employee exposure to the subject microorganism is limited at this step by the personal protective equipment worn by the process technician and the small sample volumes required. Samples are carried in sealed vials to the dedicated laboratory staff for routine analysis. Exposure to the organism is also limited during this activity by the personal protective equipment that is regularly worn by the dedicated laboratory staff (lab coat, latex or nitrile gloves, and safety glasses). All samples are inactivated with either chemical disinfectants or autoclaving prior to disposal. Due to the controls described here, handling of the fermentation samples represents minimal employee exposure.

4) Post fermentation processing & Storage –

Drying Process

Employees wear the following personal protective equipment during the drying process and cleaning operations to limit exposure to the yeast, safety glasses, gloves and uniforms.

Respirators with P100 cartridges are worn during operations with potential for exposures such as cleaning and packaging processes.

Summary of worker exposure information

Worker Activity	**Protective Equipment/ Engineering Controls**	**# of Workers Exposed**	**Maximum Duration (hrs/day)**	**Maximum Duration (days/yr)**
Seed Inoculum Transfer	Lab coat, gloves, safety glasses, hard hat			
Fermentation – samples	Lab coat and or uniforms, safety glasses, gloves, hard hat			
Drying Process	Respirators, safety glasses, gloves, uniforms			

(iv) Environmental Release and Disposal of Subject Microorganism

Possible release points for and disposal of the new microorganism during manufacture of the ethanologen *S. cerevisiae* are discussed in Section (i)(1)(ii) c) above. Efficiency of the control technology at the various potential release sites is outlined in the table below.

Control Technology Efficiency at Potential Sites of New Microorganism Release

Release Number	Amount of new substance released (CFU/batch)		Media of Release	Control Technology	Effi-ciency
	To Envi-ronment	To Control Technology			
1) Seed cryovial preparation – empty seed flasks			Water		
2) Seed inoculum transfer – empty cryovials			Land		
3) Seed inoculum transfer – empty seed flasks			Water		
4) Seed inoculum transfer – empty transfer apparatus			Water		
5) Seed Fermentor			Water		

6) Main fermentor			Water		
7) Fermentor samples			Water		
8) Fermentor off gas – release into atmosphere	(see Note)		Air (Air-borne droplets)		
9) Fermentor off gas – released into pasteurization tank			Water		
10) Drop tank for fermentor broth			Water		
11) centrifuge			Water		
12) press			Water		
13) Extrusion			water		
14 Drying			Air,water		
15) Packaging			water		
16) Ethanol production – propagation tank			land		
17) Propagation tank			water		

18) main fermentor					
8) Fermentor off gas – release into atmosphere					
19) Fermentor off gas gas – released into cook tank					
20) Fermentor samples					

Note on CFU numbers used in the above table:

Note on Fermentor off-gas:

The three potential sources for release of the new microorganism from the manufacturing facility are via solid, liquid or gaseous emissions. During normal operation, no release is expected via solid or liquid waste emissions; a negligible amount of release may occur in the fermentor off-gas and dryer exhaust.

(v) Manufacturing Site Proximity to Drinking Water Sources

The Cedar Rapids facility is located at 1000 41st Avenue Drive SW in an industrial area on the South West side of Cedar Rapids, Iowa. The primary source of Cedar Rapids' drinking water is a series of city wells within the Cedar River watershed. The Cedar River is located approximately 0.8 miles from the facility.

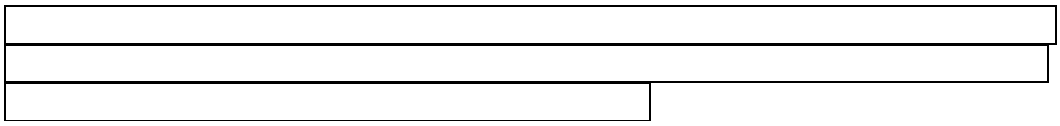
(vi) Emergency containment procedures to be followed in case of an accidental release

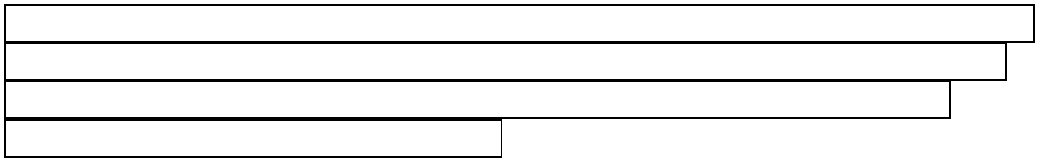
Cedar Rapids manufacturing site: In the unlikely event of a large spill of material containing the new microorganism, containment procedures will be followed. The action taken depends on the size of the spill or leak. All accidental spills and leaks

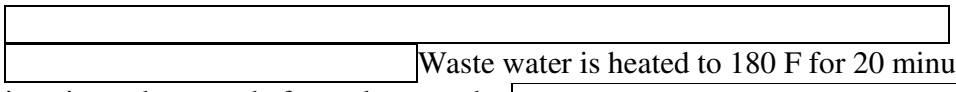
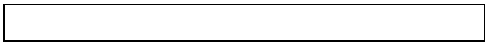
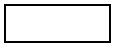
greater than ten liters of fermentation broth are reported to the supervisor and personnel are evacuated from the area for at least thirty minutes to allow aerosolized culture to settle. Before evacuation, closing any valves that are the source of leakage stops the spill or leak. For small spills, the area is treated with hypochlorite solution then cleaned up with towels; the towels are autoclaved before disposal. Larger spills require the use of two buckets, one to mop up the spill and the other for treatment with fresh hypochlorite solution. Spills larger than those that can be handled by buckets and mops are drained and flushed to the waste liquid collection system for bleach inactivation before release to the sewage treatment facility. The spill area is then treated with hypochlorite solution. The hypochlorite is left on the spill for a minimum of twenty minutes. The fermentor tank floor has a trench setup the feeds all run off to a sump. The sump feeds into a surge tank and is also the easiest way to access the surge tank. There is a manual isolation valve off of the surge tank that can be closed to contain very large spills. A drum of bleach is present at the access to the sump that supplies the surge tank in case the need to inactivate a spill arises. An incident report is completed for spills greater than or equal to 10 liters.

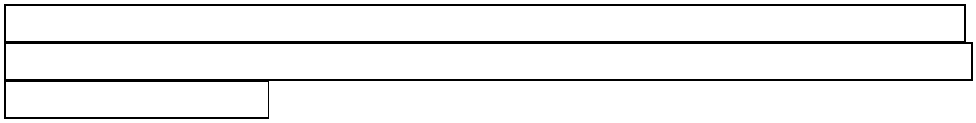
If spills are sent to the drain, the aqueous waste will go to the respective Water pollution Control facilities of the City of Cedar Rapids. Discharge would be indirect to surface waters. The Water Pollution Control facilities will be notified, if the fermentation spill volume exceeds 10% of normal discharge.

Any spills during the drying process go to a contained sump that is pumped to a tank for heat inactivation prior to discharge to the city waste water facility.






Waste water is heated to 180 F for 20 minutes to inactivate the yeast before release to the 




(vi) Procedures for disposal of any articles, waste, clothing, or other equipment in the activity, including procedures for inactivation of the new microorganism, containment, disinfection, and disposal of contaminated items

The procedures requested here are described above.

(2) For sites not controlled by the submitter

The new microorganism will be used for commercial ethanol manufacture at customer sites. Below is a description of how the modified ethanologen (*S. cerevisiae*) will be handled at a potential customer ethanol plant.

All production activities are conducted in a building or tanks and there is no intentional testing outside of the structure. Buildings enclose most of process except the fermenters are located partially outside of the building enclosure. Access to the building and processing areas is controlled.

In general, the process containing viable yeast is enclosed and the majority of waste streams are recirculated back into the process into the beerwell or slurry tanks and ultimately back into the fermenters. Any viable yeast released into waste streams is re-circulated back into the process and inactivated in the distillation column. The temperature of the cook tank and whole stillage tank (after distillation) are

The yeast is received in vacuum sealed Mylar bags from DuPont. Yeast is added to a blend tank or at the manway on top of the propagator (seed fermenter). Yeast is slurried with water and mash in a closed tank. Air is exhausted to the centrate tank and then to the thermal oxidizer (760ºC). After addition to the propagator, all subsequent material transfers containing viable yeast are through enclosed lines. From the propagator, the broth is transferred through closed lines to the large fermenters. Air from the fermenters exhaust through the CO2 scrubber The scrubber water flows to the cook tank

Fermentation broth is then transferred to the beerwell (holding tank) which then is transferred to the beer column to remove the ethanol. Residual yeast material at the bottom of the beer column is transferred to the whole stillage tank which is maintained at >80ºC. The ethanol is processed through 2 additional distillation steps, the rectifier column and finally to the molecular sieve column.

The whole stillage is centrifuged and the solids are dried (>480ºC) to produce dried distiller grains. The thin stillage from the centrifuge is evaporated (>90ºC) to make syrup which is added the solids in the dryer.

Trenches inside the building flow to a sump which is pumped into the beerwell (recycled into the process). All wash downs and spills are collected in the trench. Samples are also poured into the trench. All lines, tanks and processing equipment are cleaned with a hot CIP solution (>80ºC).

Employees change into uniforms each shift and do not take or wear the uniforms off-site. Uniforms are laundered by a professional service. Employees also wear safety glasses, gloves and protective shoes. Furthermore, employees receive training by the Technically Qualified Individual on the potential health hazards of the microorganism, work practices, inactivation procedures, equipment and room cleaning and spill clean-up.

(j) Health and Environmental Effects Data

(1) Test data on the new microorganism – include all test data which are related to the effects on health or the environment of any manufacture, processing, distribution in commerce, use, or disposal of the new microorganism

DuPont Industrial Biosciences has not conducted tests specifically on the new microorganism, but has conducted numerous tests on [redacted]

The non-toxic and non-pathogenic status of *S. cerevisiae* is well established (see (j)(2)(i) below). The new microorganism *S. cerevisiae* [redacted] will be contained during use and inactivated prior to disposal.

(2) Full report or standard literature citation for test data:

(i) Health effects data

Saccharomyces cerevisiae is by far one of the mostly used microorganisms; having been used for hundreds of year in food applications such as baking and brewing. *S. cerevisiae* has been tested regarding potential pathogenicity and toxicity, and products derived from various strains have been investigated for numerous toxic endpoints. DuPont Industrial Biosciences have not conducted any company studies (in-house or by contractor) to assess the pathogenicity and toxicity of the production strain being subject to this risk assessment.

However, a thorough integrated risk assessment has been conducted by the U.S. Environmental Protection Agency (EPA 1997). Based on the recommendation of this publication, the US EPA subsequently added *S. cerevisiae* to the Tier I exempt list (40 CFR 725.420) under the Microbial Products of Biotechnology; Final Regulation Under the Toxic Substances Control Act section 5 (62 FR 17910).

Moreover, The Food and Drug Administration (FDA) rates *S. cerevisiae* extract as GRAS (see e.g. FDA 2002, 2003, 2006, 2011, 2012).

The main conclusion for hazard assessment regarding human health from the U.S. EPA risk assessment is that *S. cerevisiae* is an organism which has an extensive history of safe use. Despite considerable use of the organism in research and the presence of *S. cerevisiae* in food, there are limited reports in the literature of its pathogenicity to humans or animals, and only in those cases where the subject had a debilitating condition. Factors associated with the virulence of yeasts (i.e., phospholipases) indicate that this organism is non-pathogenic. The organism has not been shown to produce toxins to humans (EPA 1997).

It is concluded that the new microorganism, *S. cerevisiae* strain [ ] is non-pathogenic and non-toxigenic.

(ii) Ecological effects data

The genetic modifications introduced into *S. cerevisiae* [ ] are not expected to significantly affect the survival of the organism. Several survival studies on *S. cerevisiae* comparing wild-type yeast and genetically modified yeast conclude that the genetically modified variants do not survive better than the wild-type (Broker 1990, Fujimura *et al.* 1994, Ando *et al.* 2005, Valero *et al.* 2005).

(iii) Physical and chemical properties data

There are no applicable physical and chemical properties data for the subject microorganism.

[ ]

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(iv) Environmental fate characteristics

See (j)(2)(ii) above.

(v) Monitoring data and other test data related to human exposure or to environmental release of the new microorganism

No additional data are available for this particular microorganism. However, the risk assessment conducted by the U.S. EPA provides a general overview of *S. cerevisiae* and its safety as an industrial production organism (EPA 1997).

(3) Other data concerning the health and environmental effects of the new microorganism that are known to or reasonably ascertainable to the submitter

(i) Any data, other than test data, in the submitter's possession or control

None other than that cited above.

(ii) Any data, including test data, which are not in the submitter's possession or control

None other than that cited above.

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Broker, M. (1990). A study on the survival of wild-type, laboratory and recombinant strains of the baker yeast *Saccharomyces cerevisiae* under sterile and nonsterile conditions. Zentralblatt Fur Hygiene Und Umweltmedizin 190:547-557.

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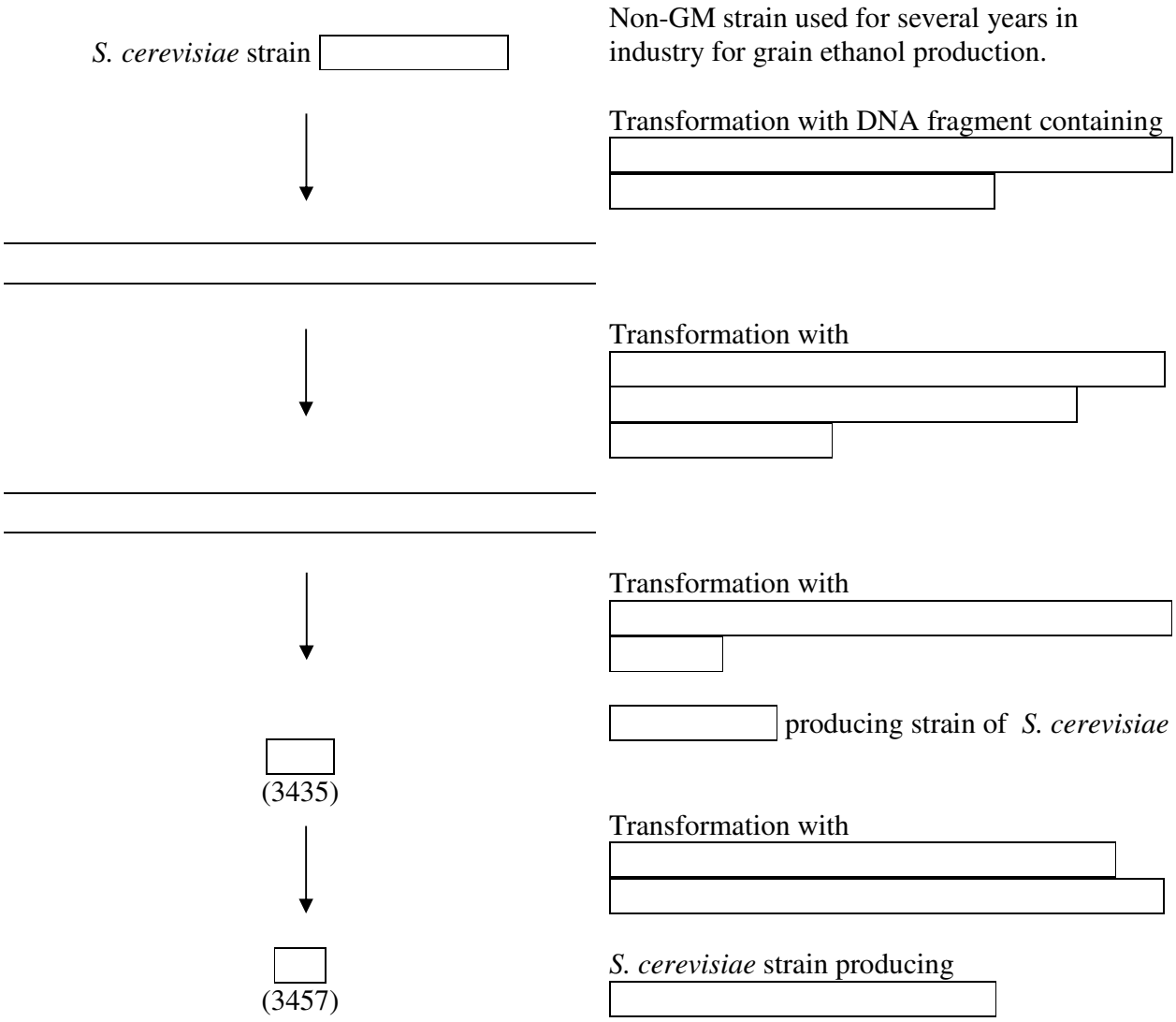
Fujimura, H., Sakuma, Y. and Amann, E. (1994). Survival of genetically-engineered and wild-type strains of the yeast *Saccharomyces cerevisiae* under simulated environmental-conditions - a contribution on risk assessment. Journal of Applied Bacteriology *77:689-693.*

Sherman, F. (2002). Getting started with yeast. Guide to Yeast Genetics and Molecular and Cell Biology, Pt B 350:3-41.

Valero, E., Schuller, D., Cambon, B., Casal, M. and Dequin, S. (2005). Dissemination and survival of commercial wine yeast in the vineyard: A large-scale, three-years study. Fems Yeast Research 5:959-969.

List of Appendices

Appendix 1: Scheme of Production Strain Construction



Appendix 2. Host strain identity

Identification of [redacted] host strain [redacted] as *Saccharomyces cerevisiae* via 18S ribosomal DNA sequence alignment

A 1977 bp region encoding 18S ribosomal RNA was amplified from genomic DNA of strain [redacted] the host strain used to create the microorganism, *S.cerevisiae* [redacted]) and strain *S. cerevisiae* [redacted] using primers [redacted]

[redacted]

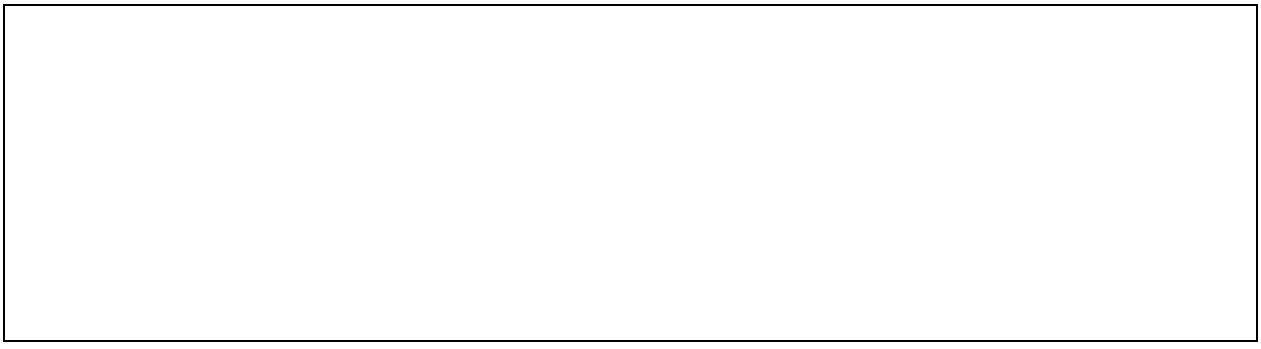
The purified PCR products were sequenced with primers [redacted]

[redacted]

Contigs were generated from the quality-trimmed overlapping sequence reads. Then, the 1805 bp consensus contig sequences were aligned to the corresponding genomic DNA regions of reference *S. cerevisiae* strain S288c (GenBank Accession# NC_001144, Chromosome XII:465072-466876).

In addition, alignments made to the corresponding genomic DNA regions of related *Saccharomyces* species, *S. bayanus* (GenBank Accession# AACA01000199, 1336-3147), *S. castellii* (GenBank Accession# AACF01000230, 1884-3687) and *S. mikatae* (GenBank Accession# AABZ01000315, 5136-6940).

An overall alignment is provided below. There were no differences among the sequences of the reference *S. cerevisiae*, the [redacted] strain, and strain [redacted]. In contrast, alignments with the most closely related *Saccharomyces* species revealed one or more base differences (highlighted below) in the sequence of the 18S ribosomal RNA gene. Overall, this confirms the identity of the recipient strain as *S. cerevisiae*.



Appendix 3. Detailed Strain Construction Scheme

[redacted]

[redacted]

[redacted]

[redacted]

[redacted]

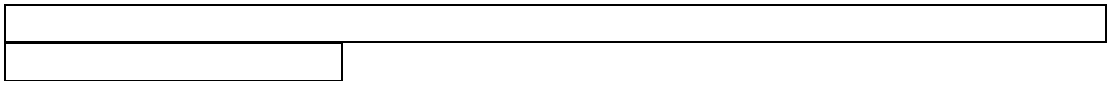
[redacted]

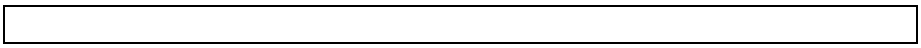
[redacted]

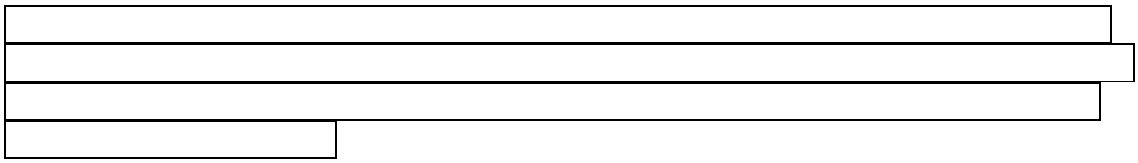
[redacted]

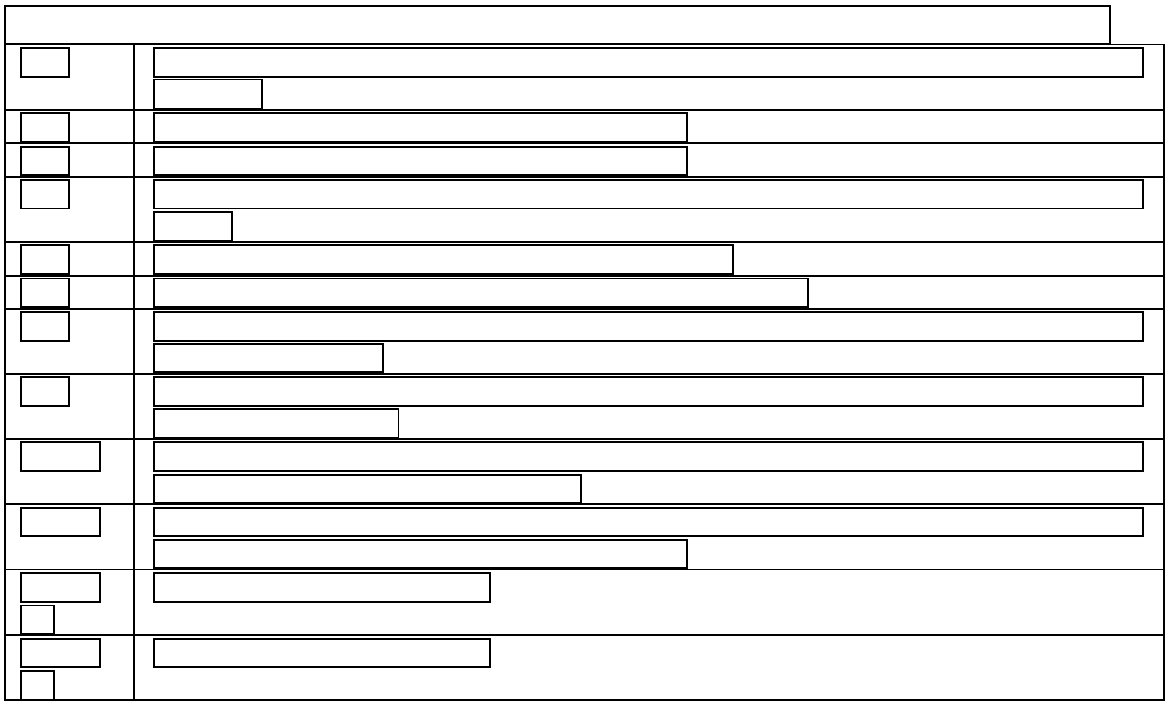
[redacted]

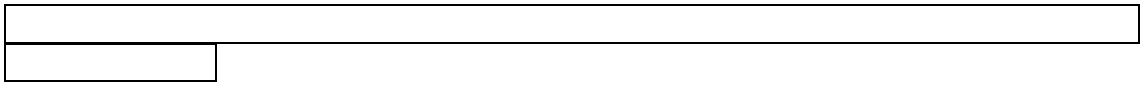
1 [redacted]

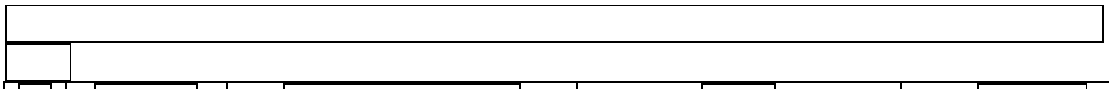


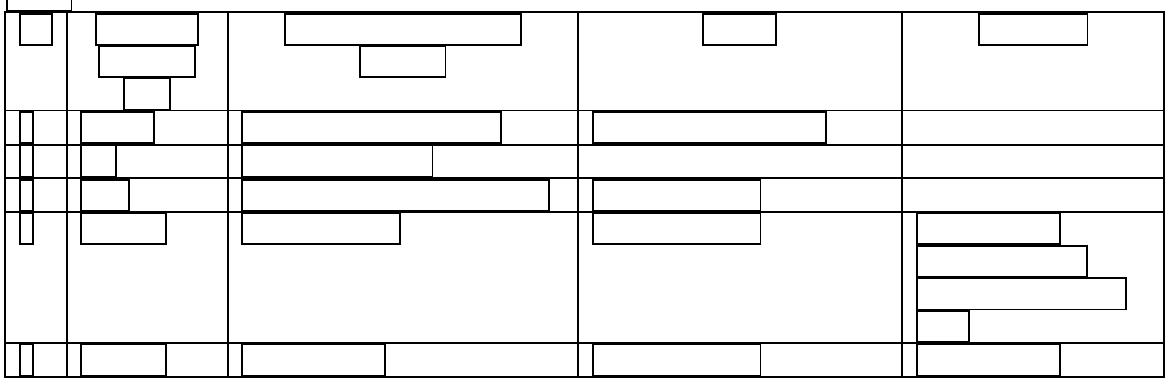


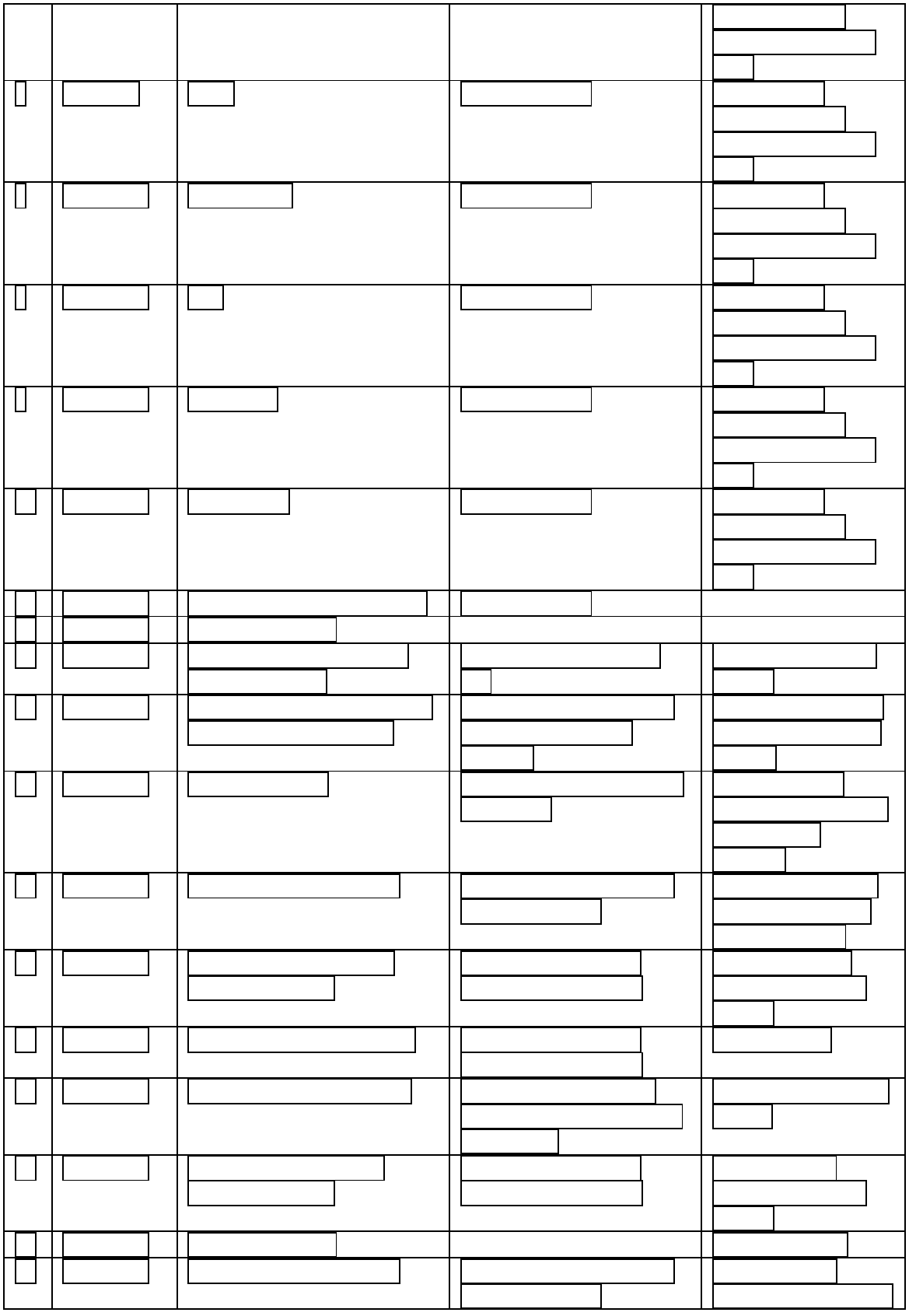


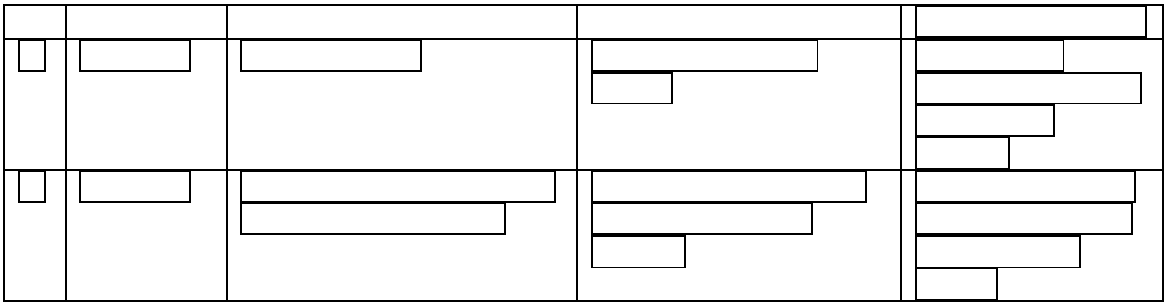


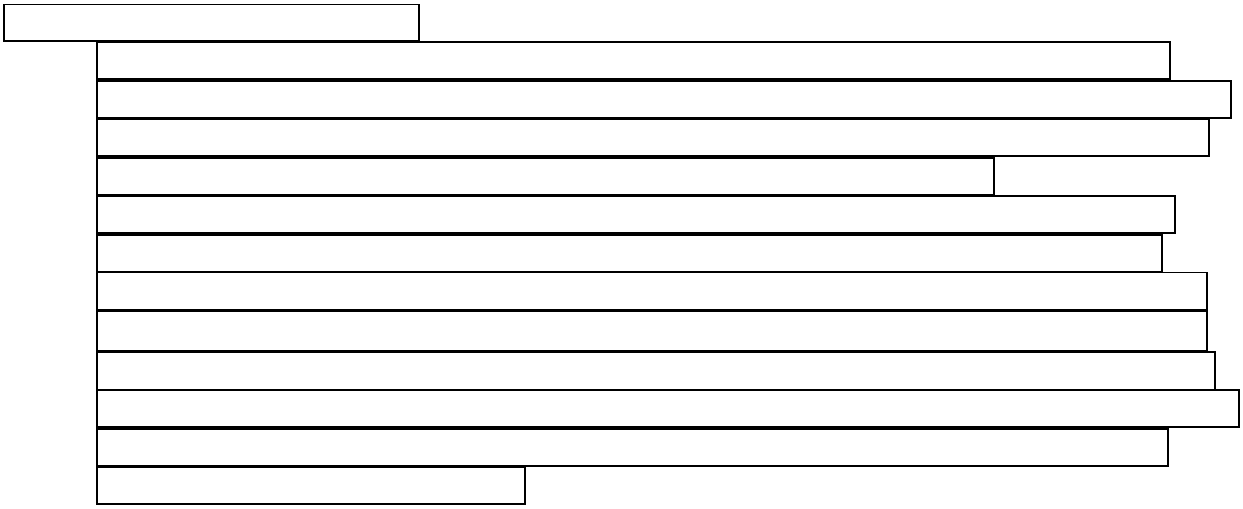


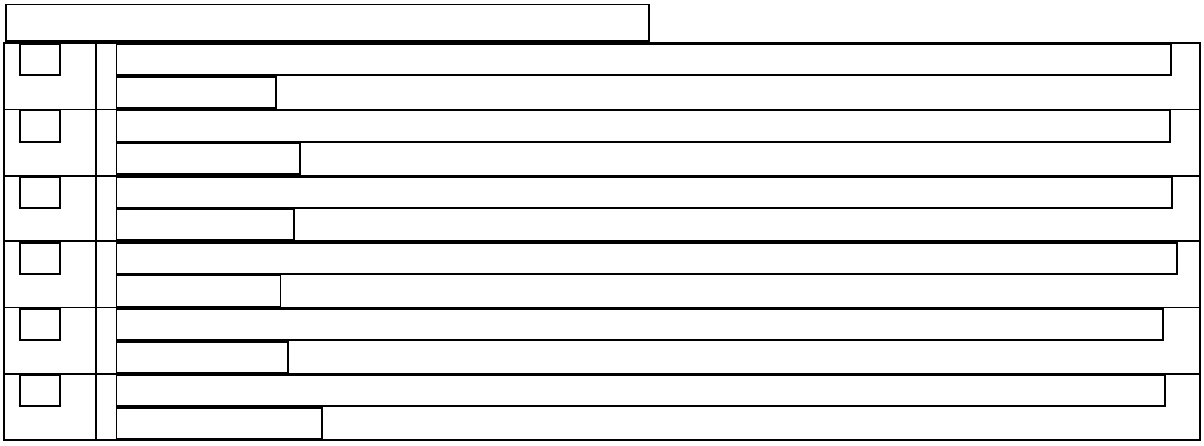


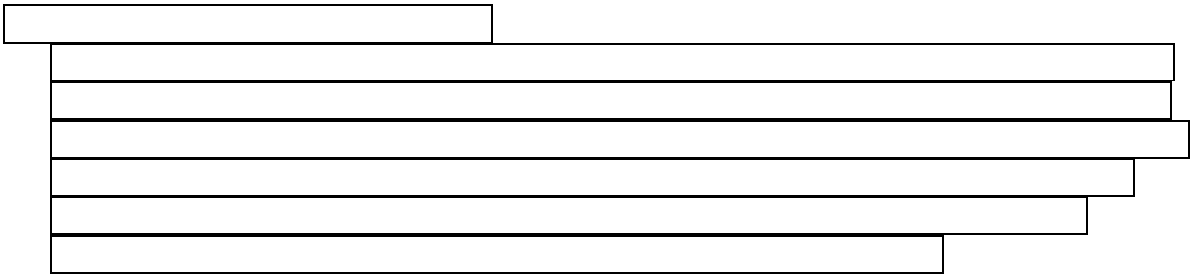


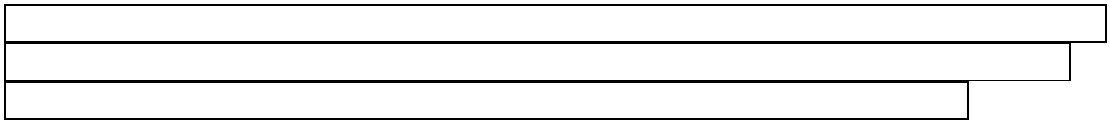


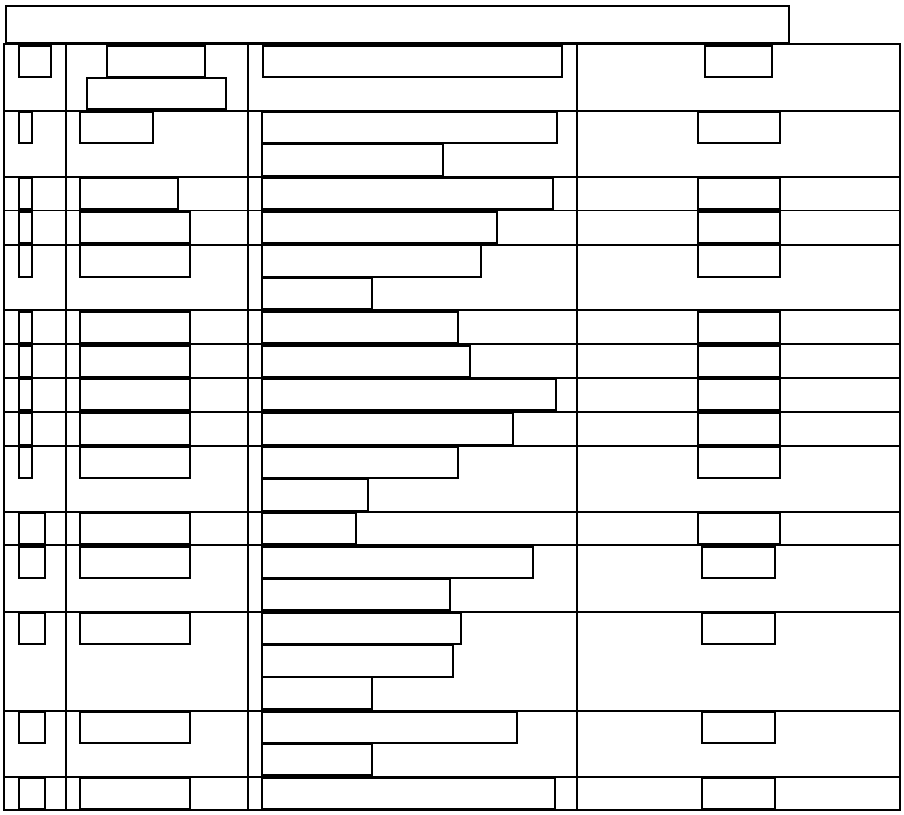


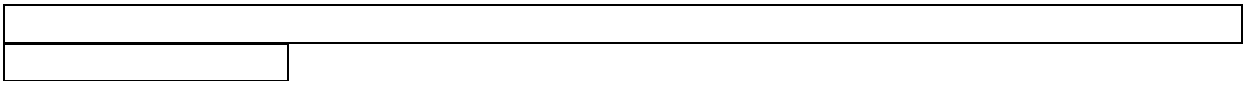


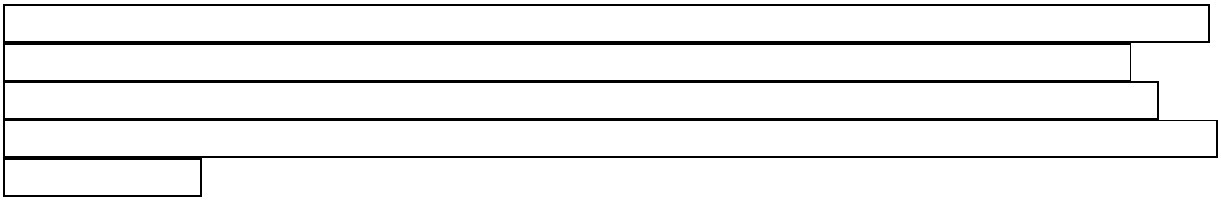


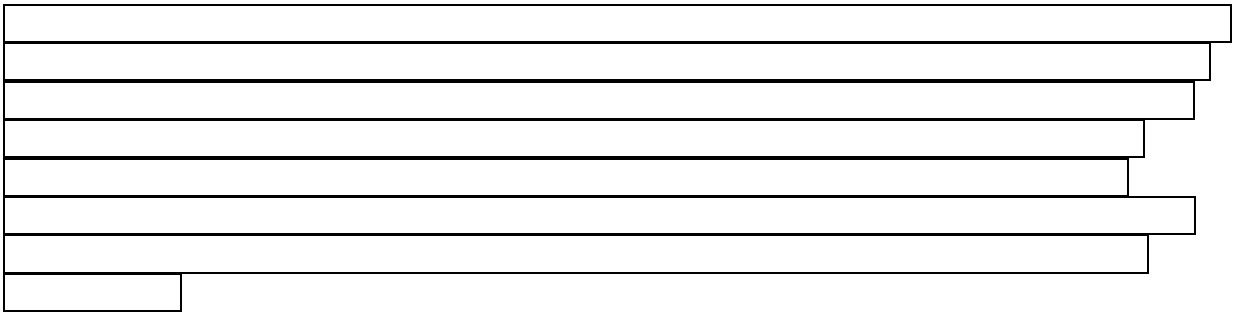


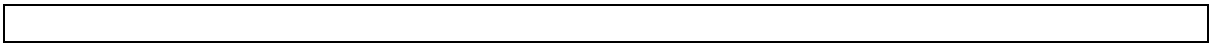


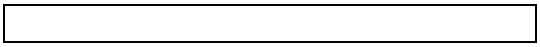


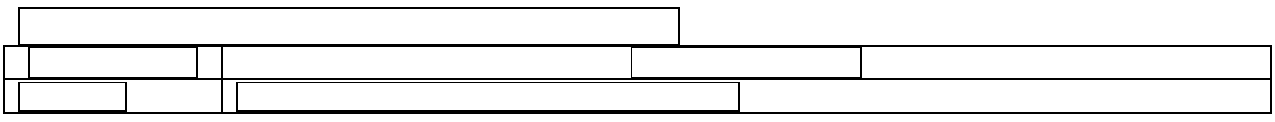


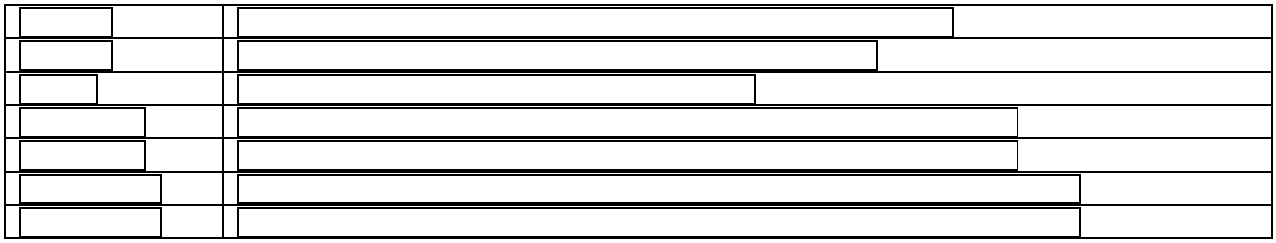


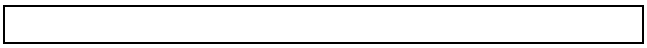


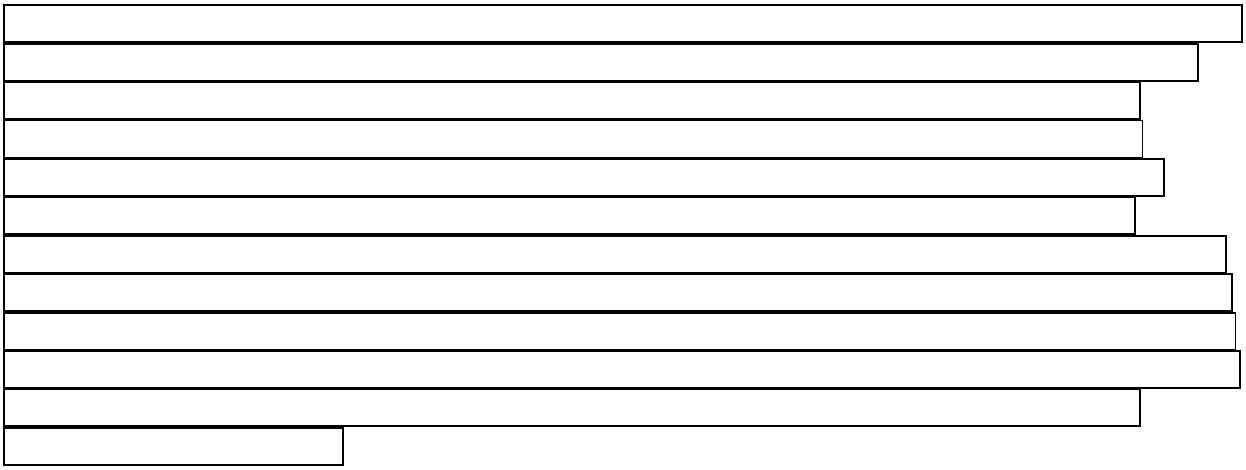


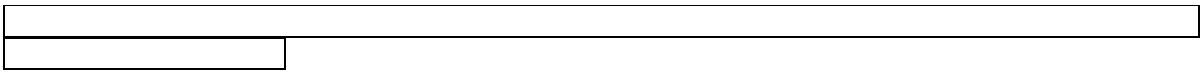












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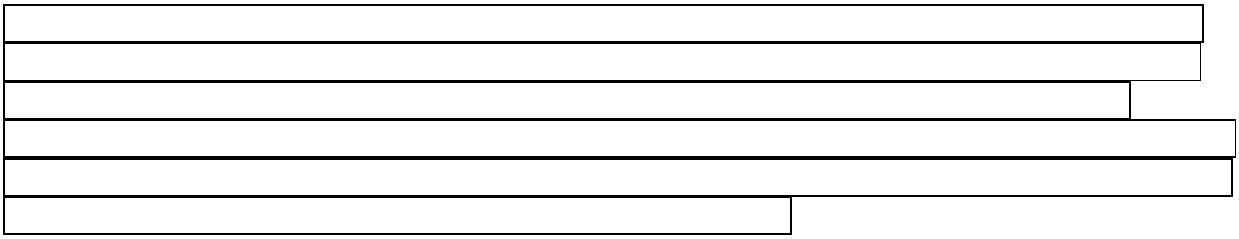

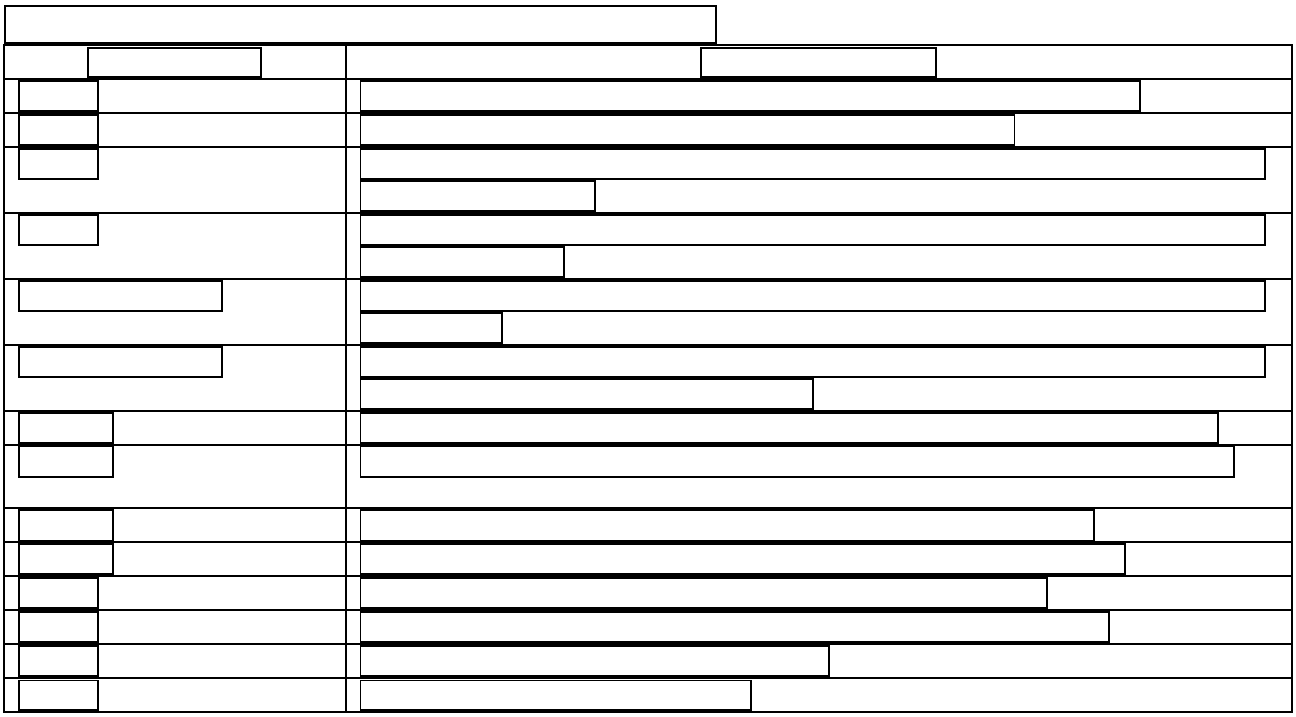

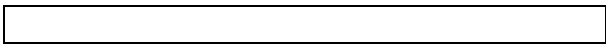

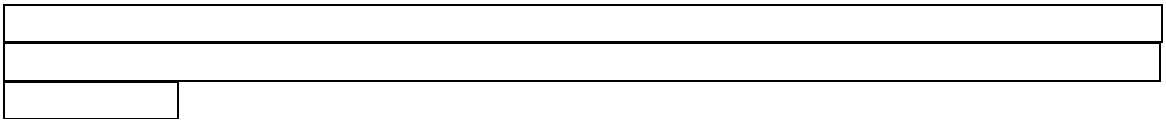

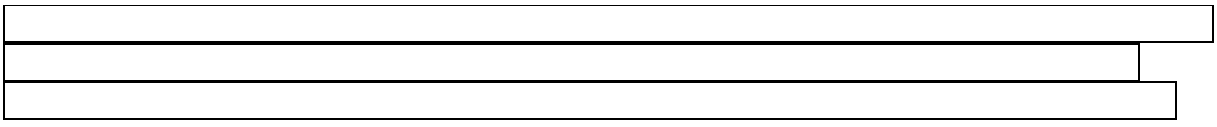

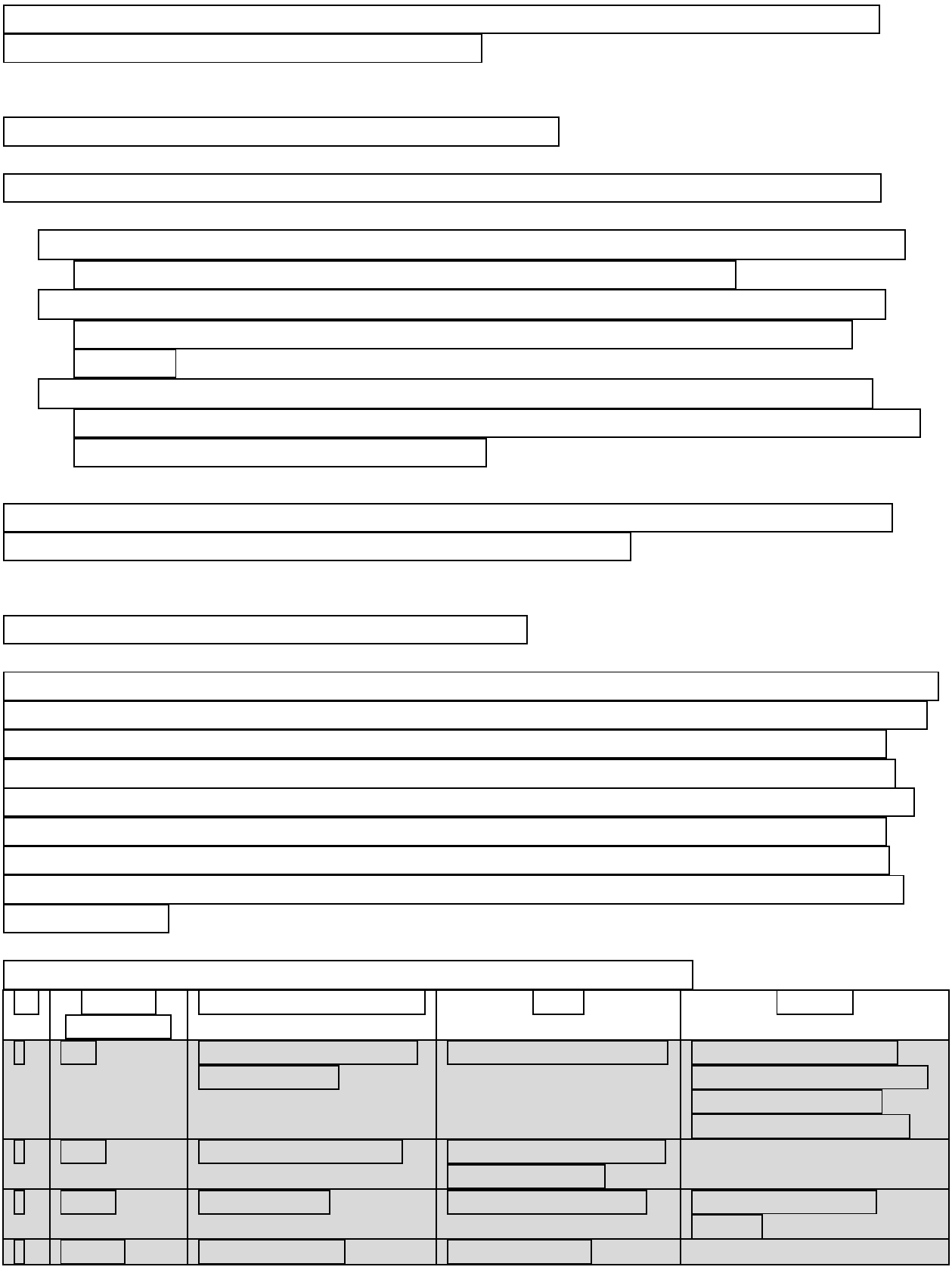

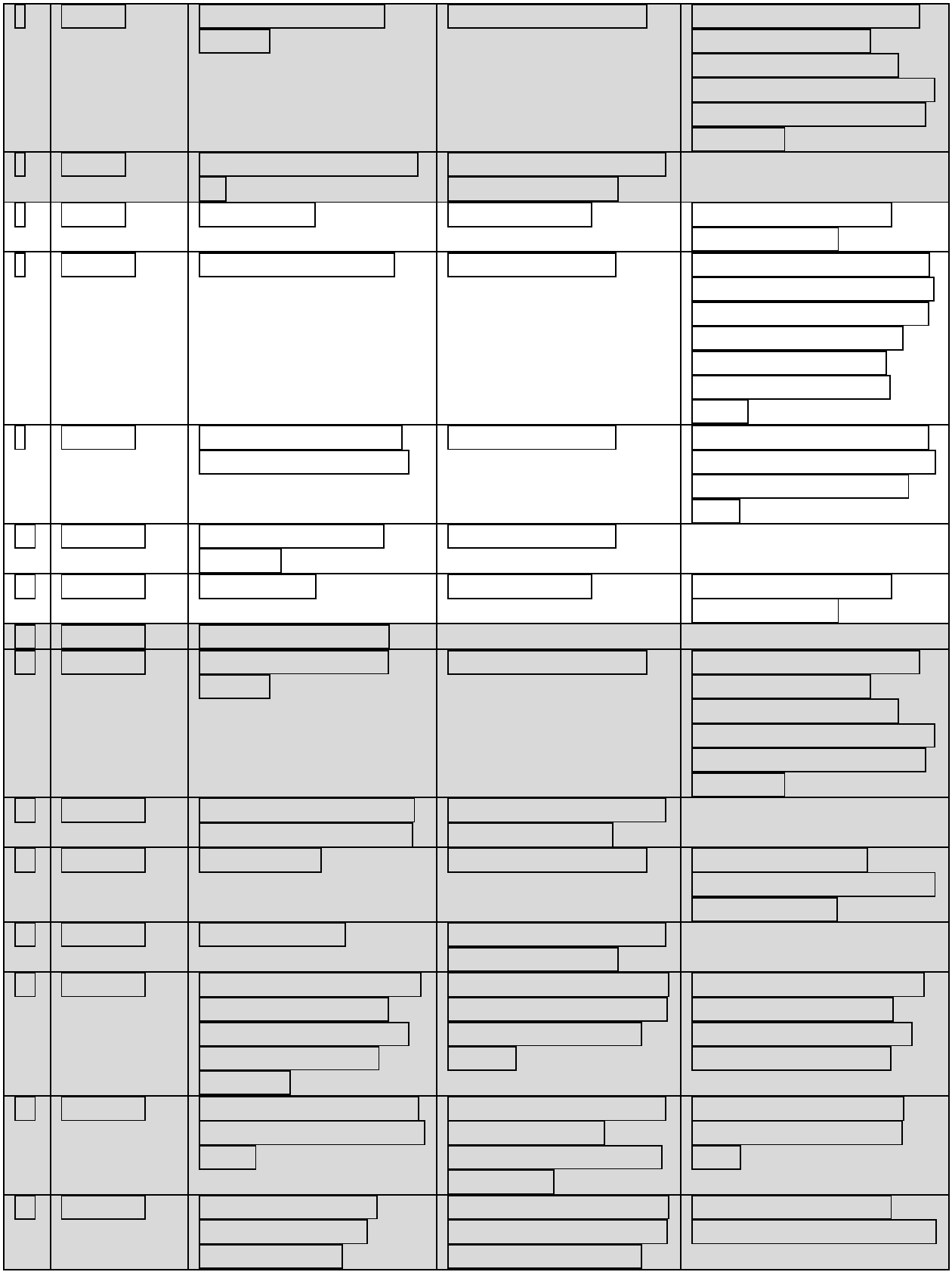

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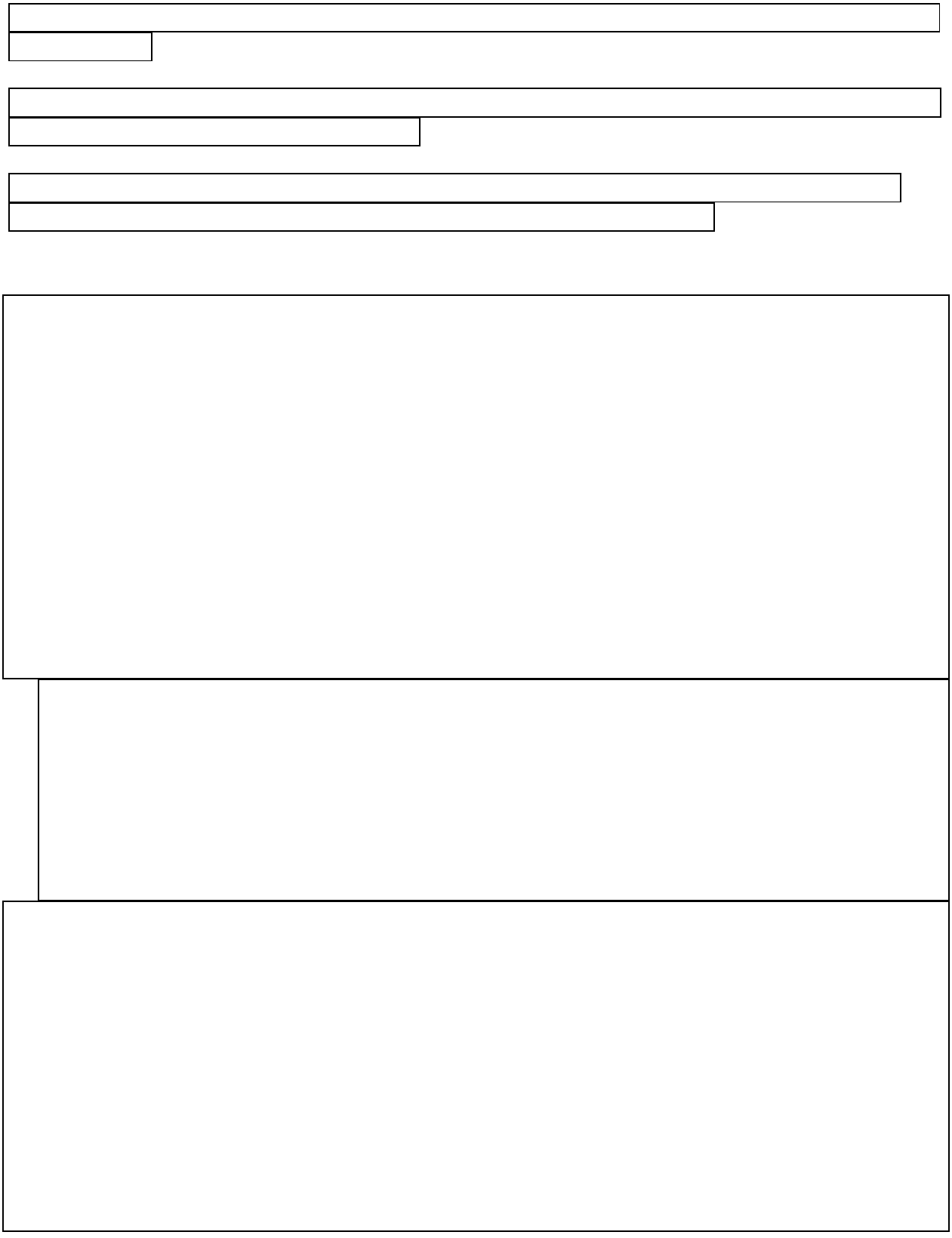

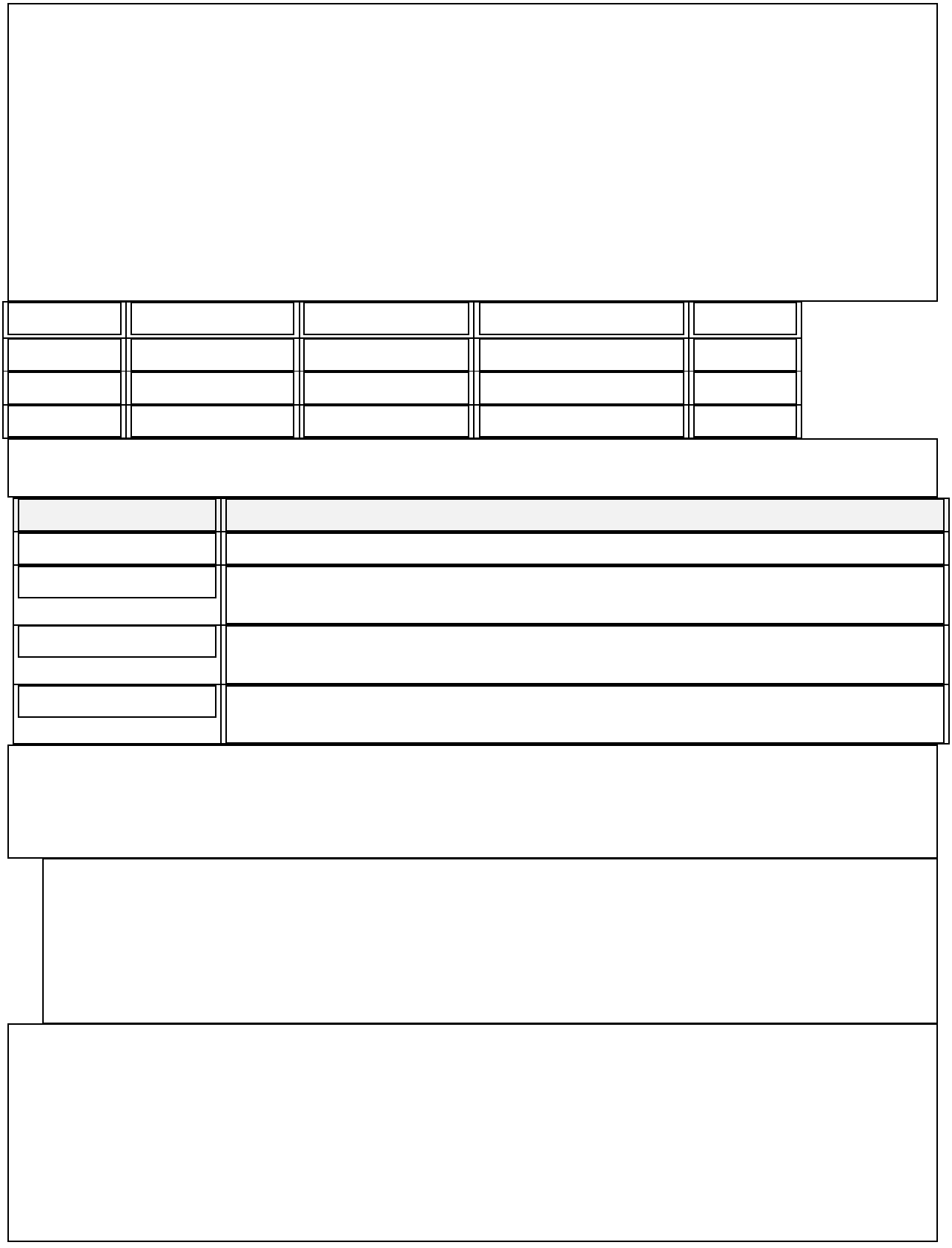

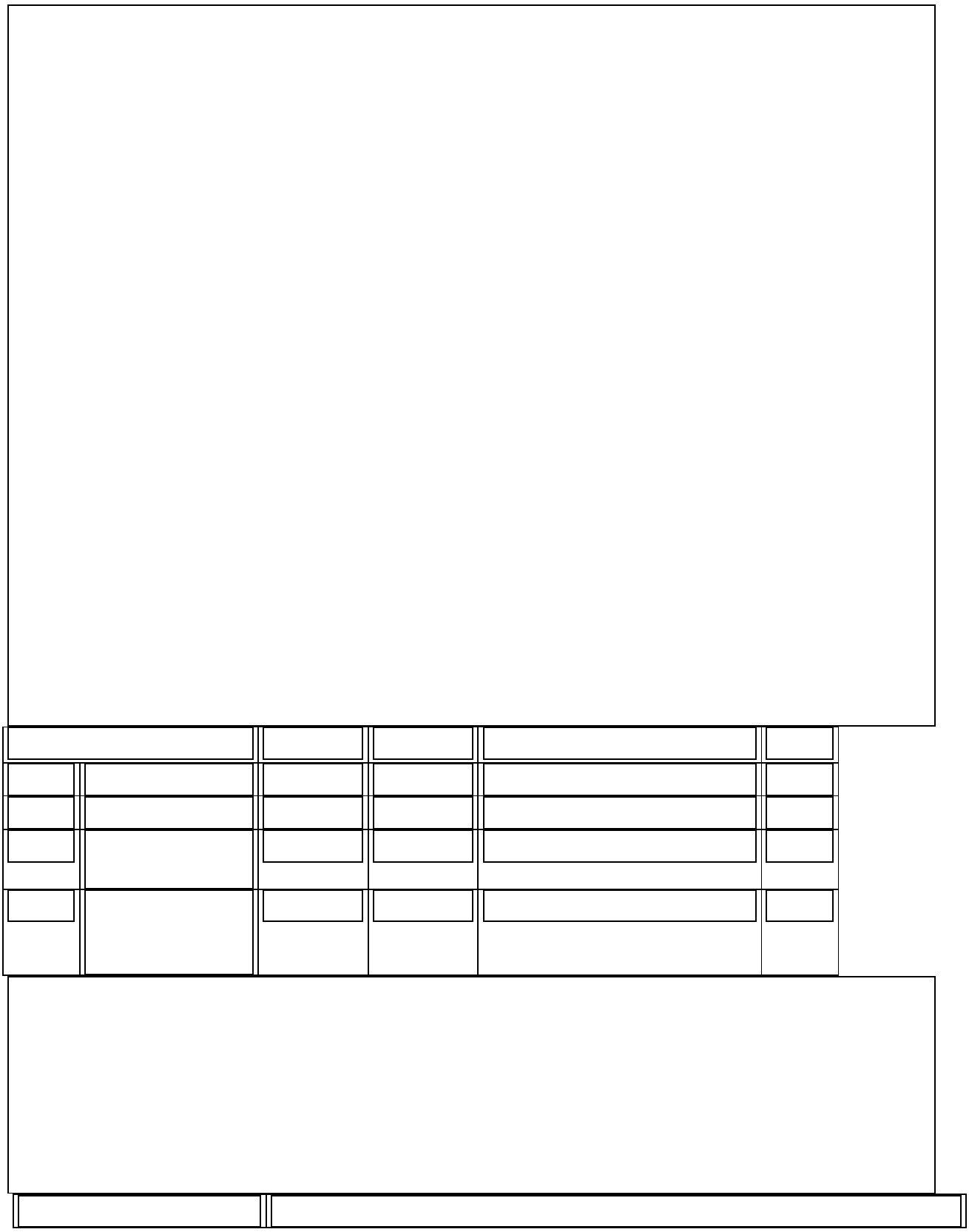

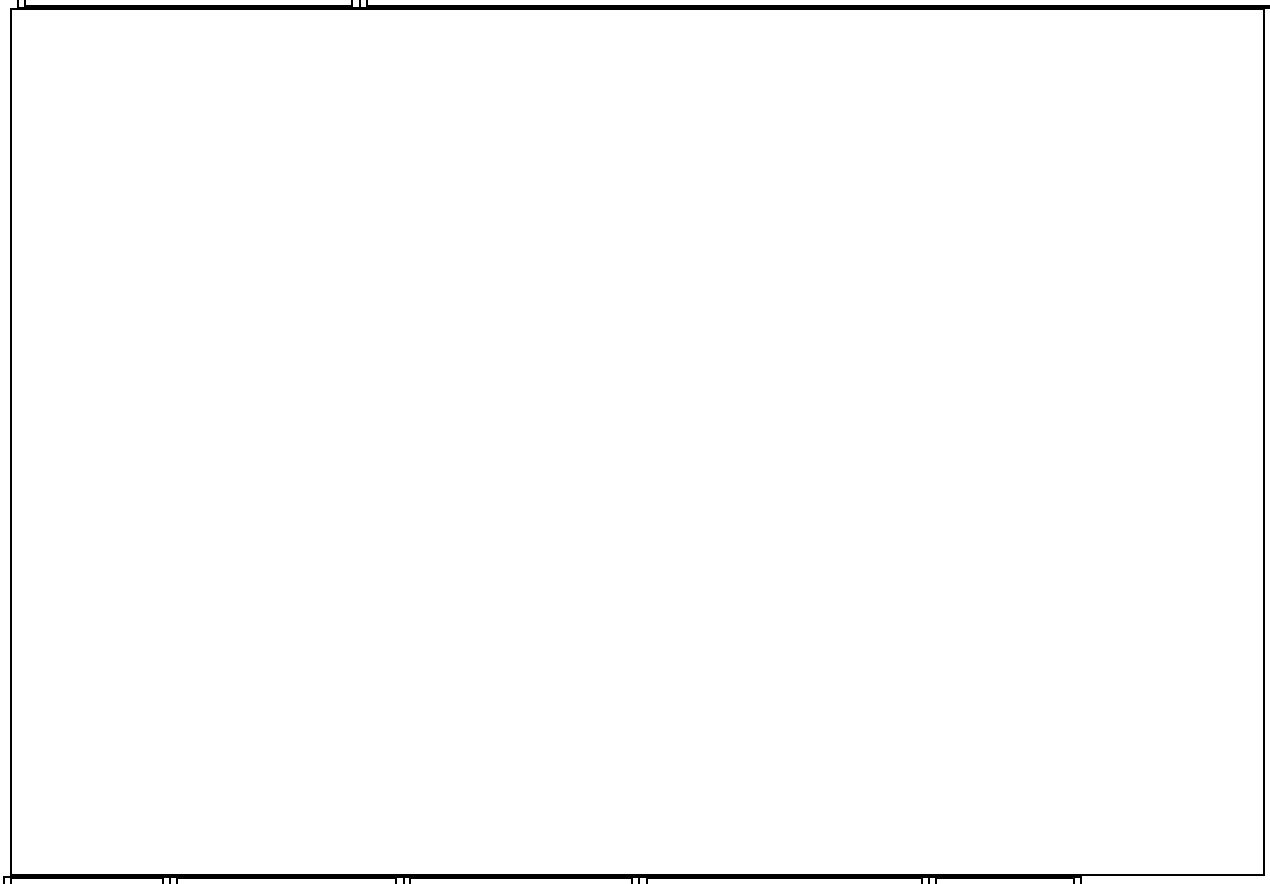

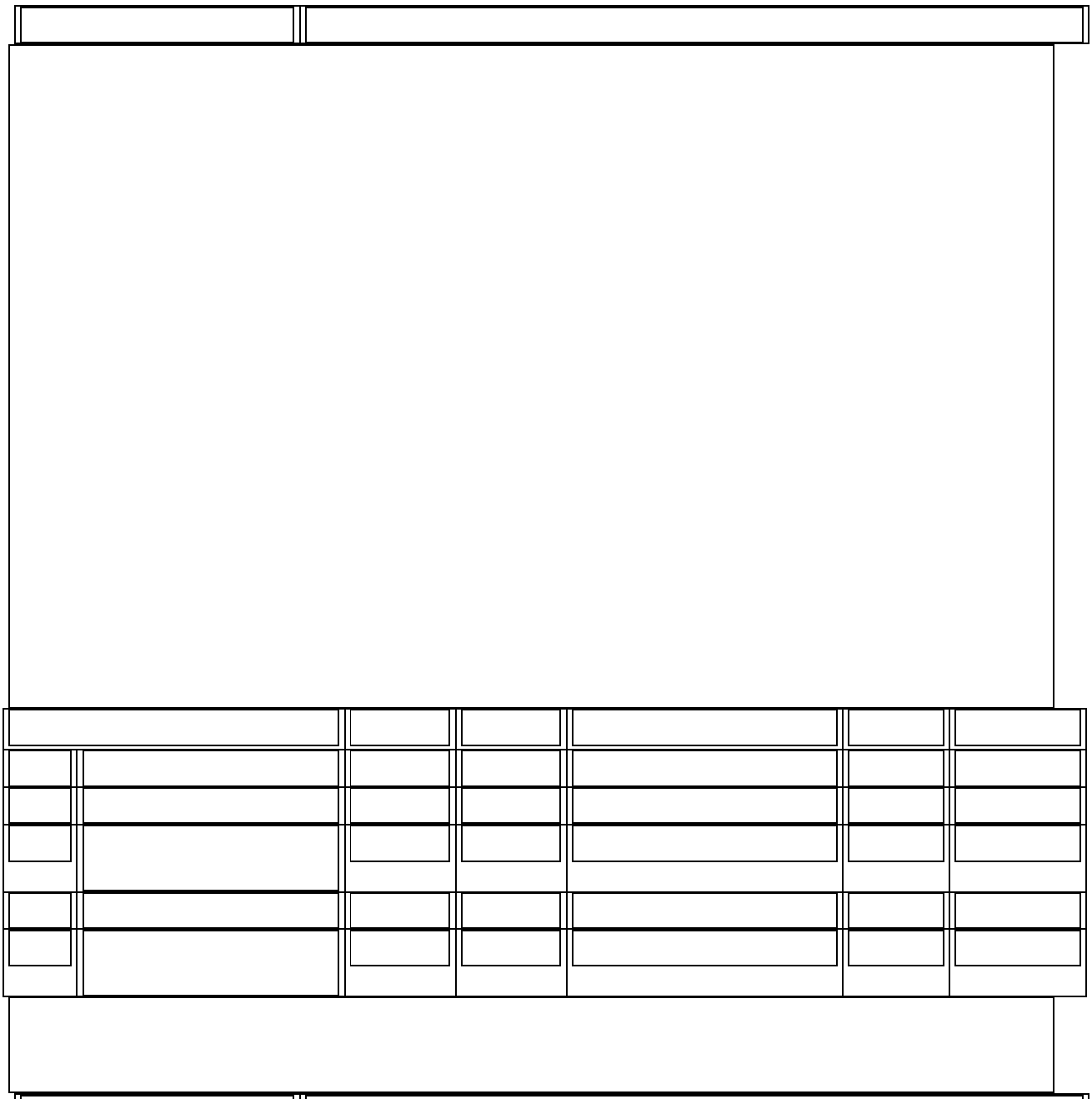

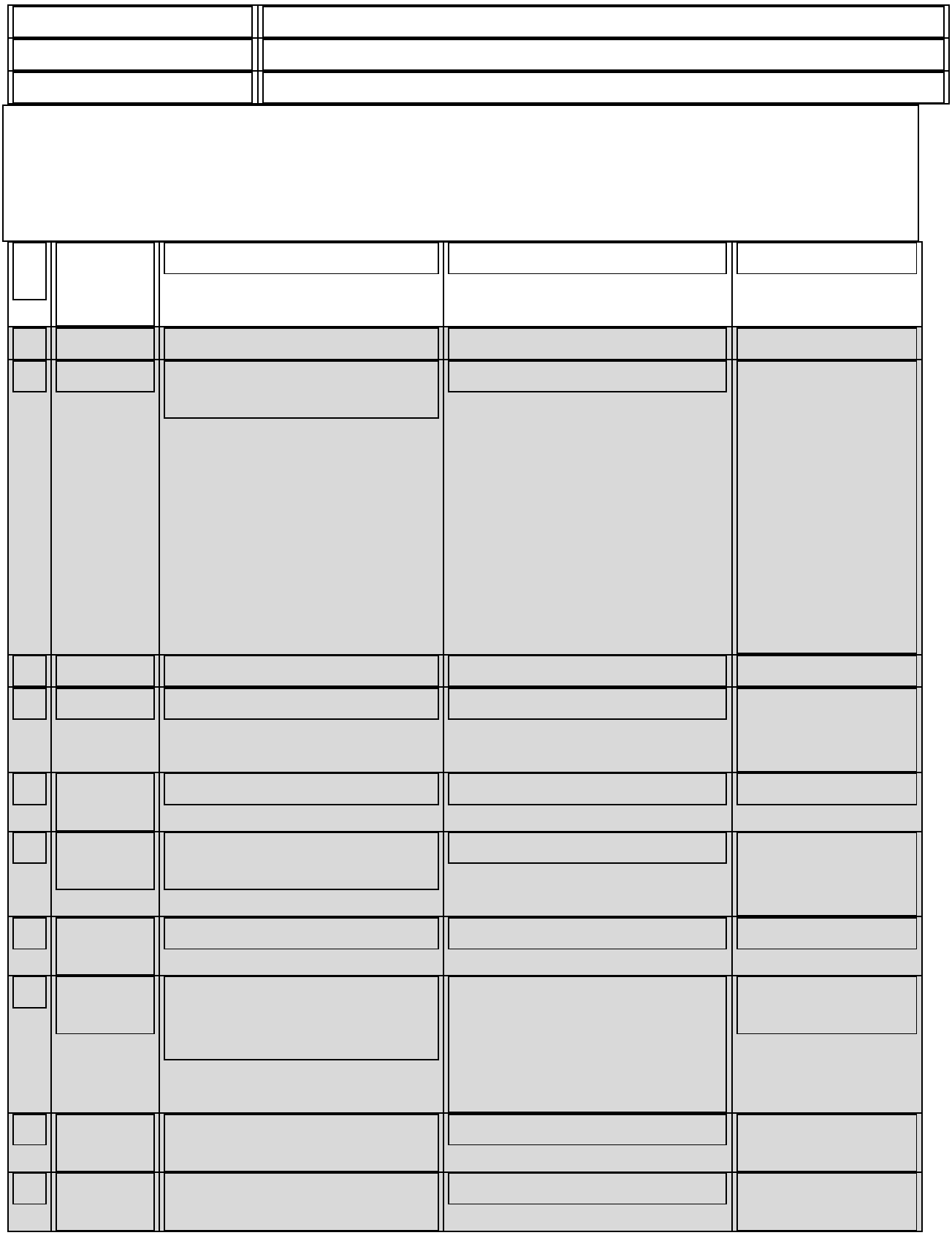

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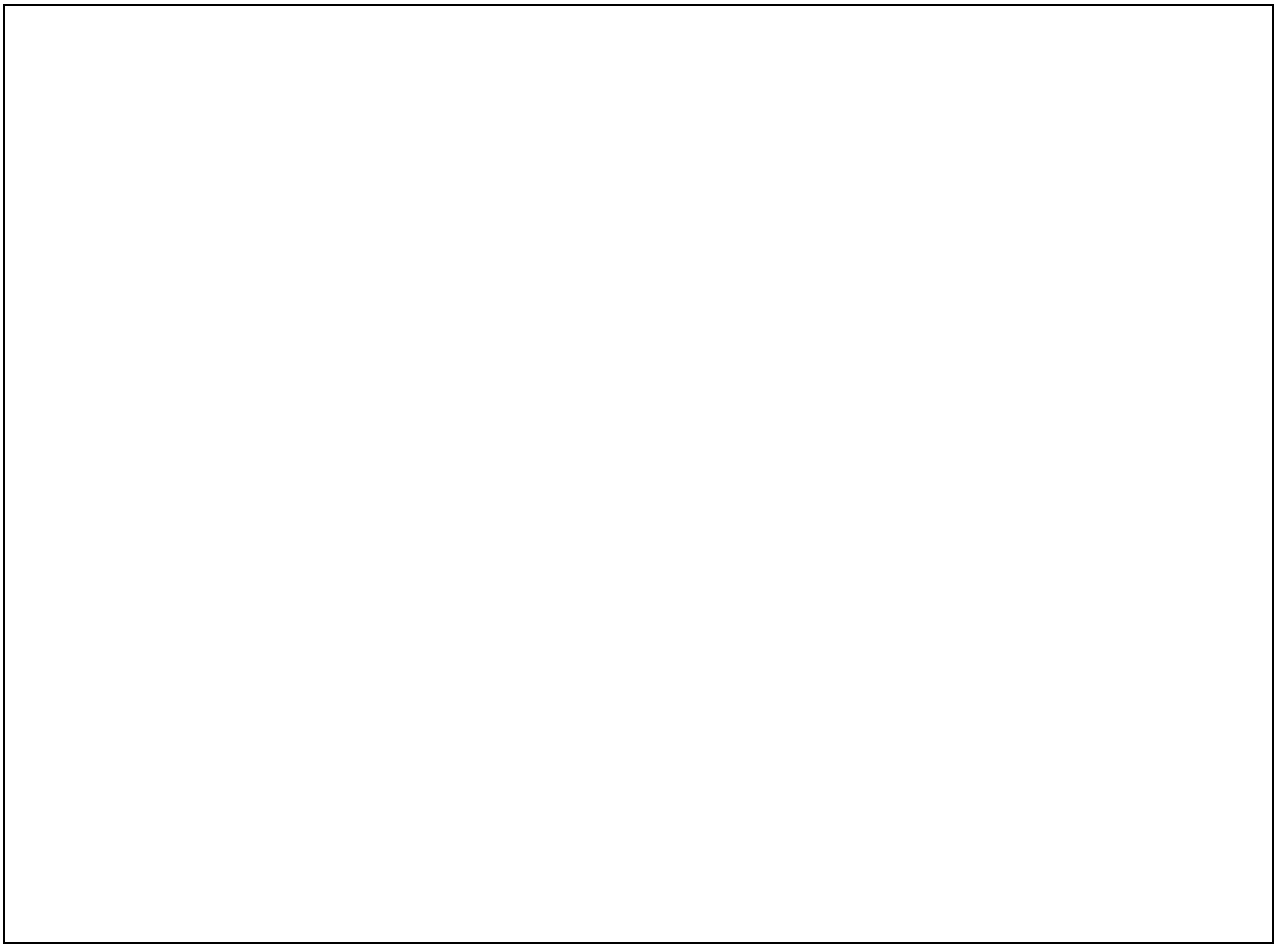

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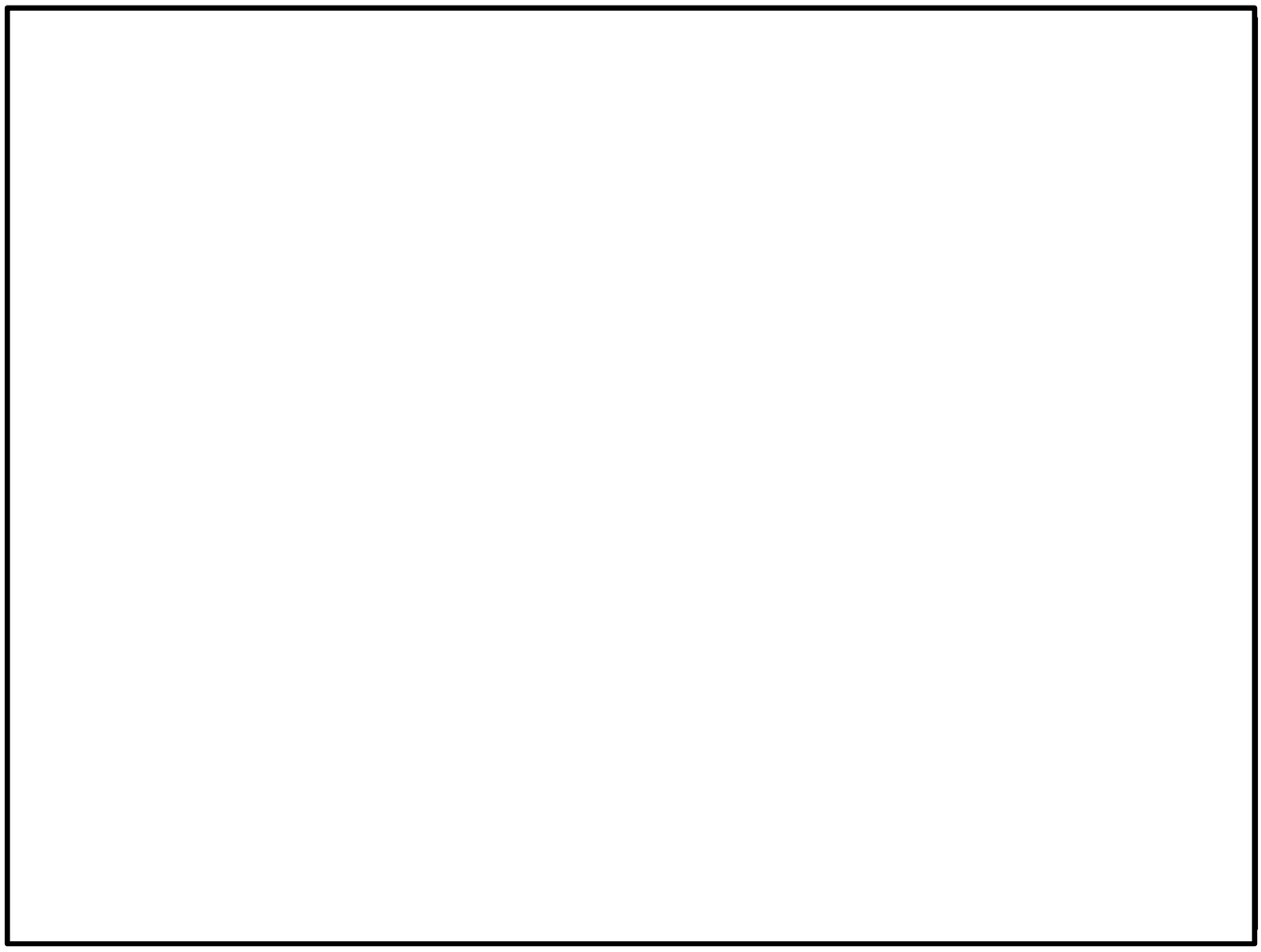

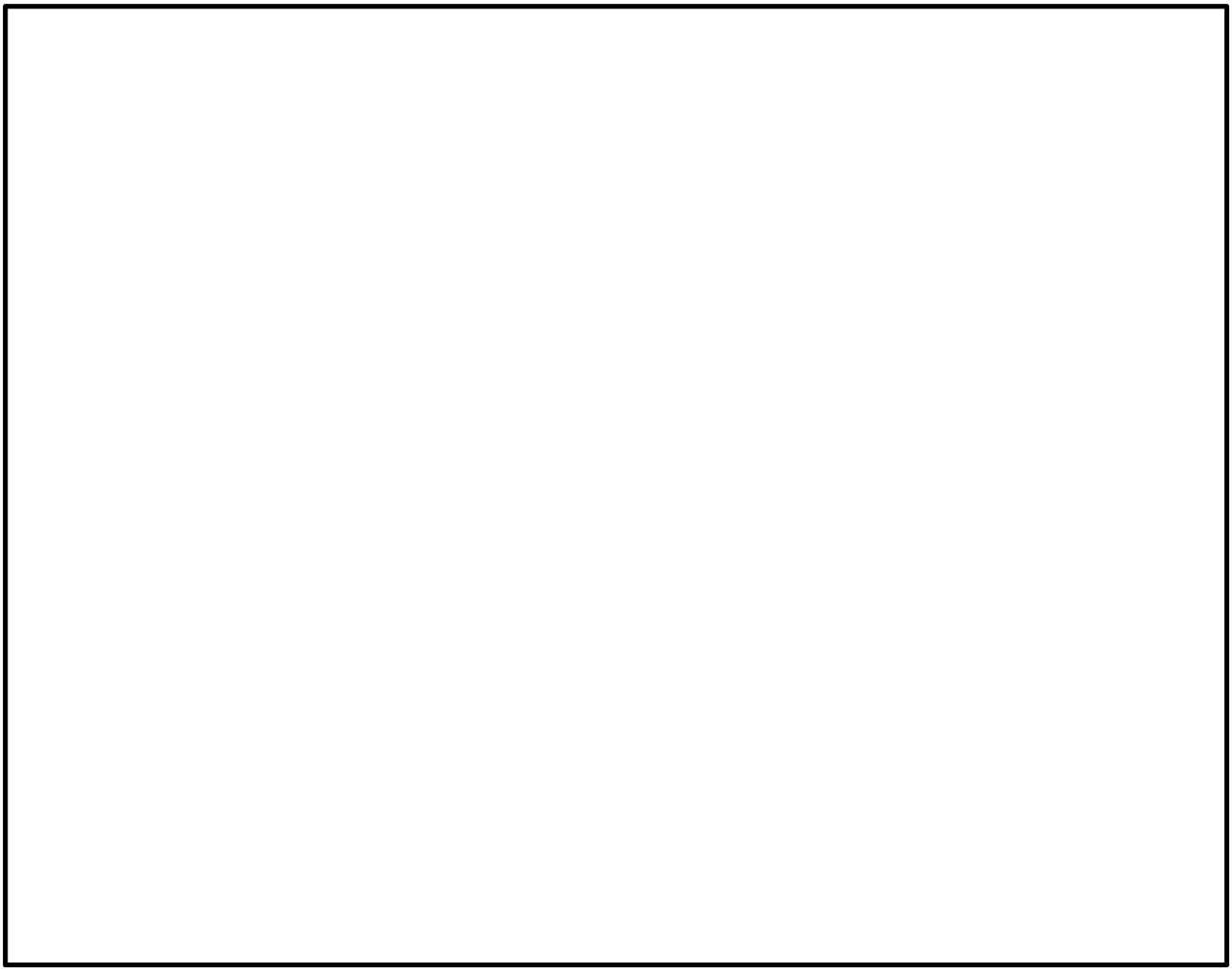

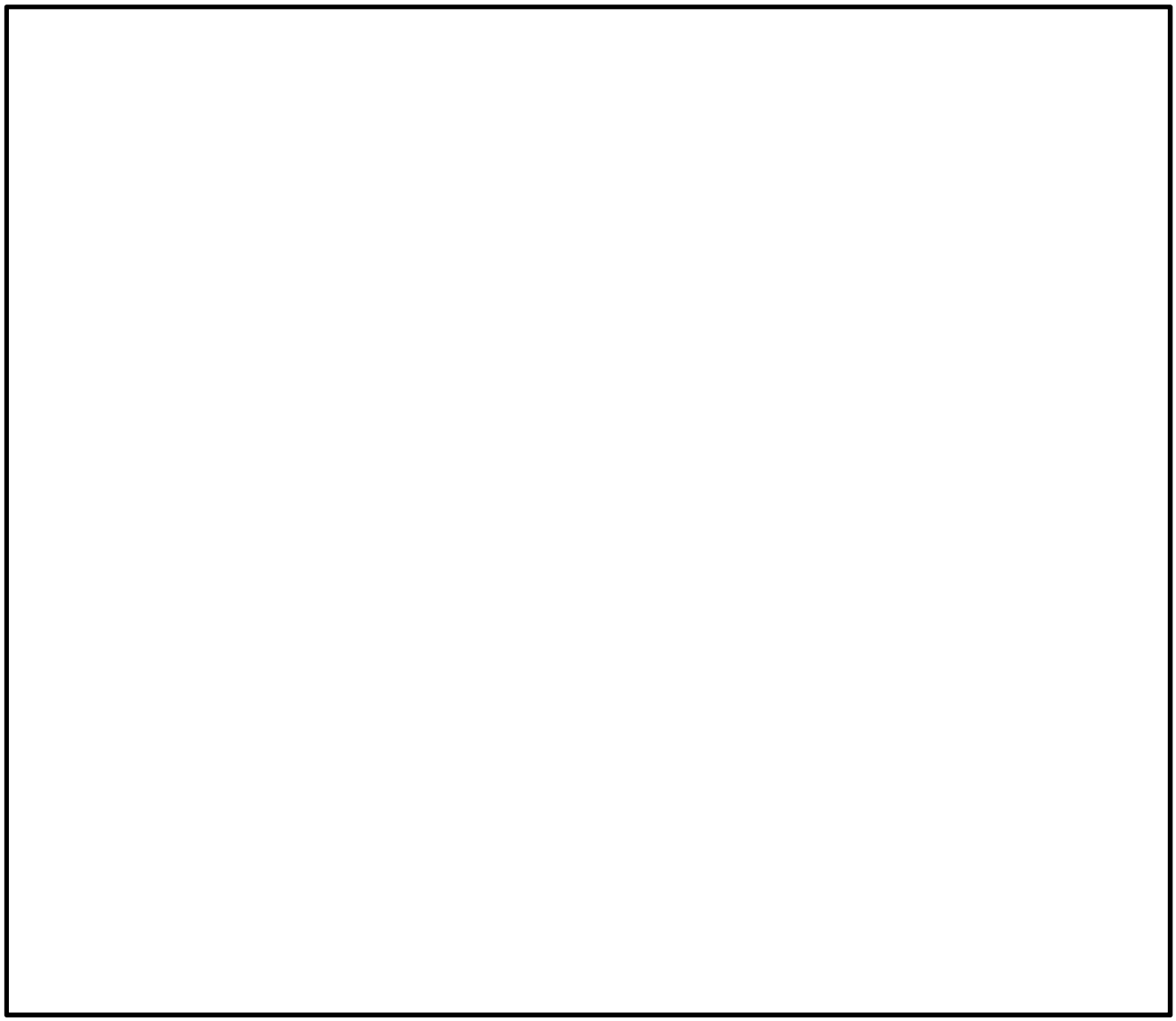

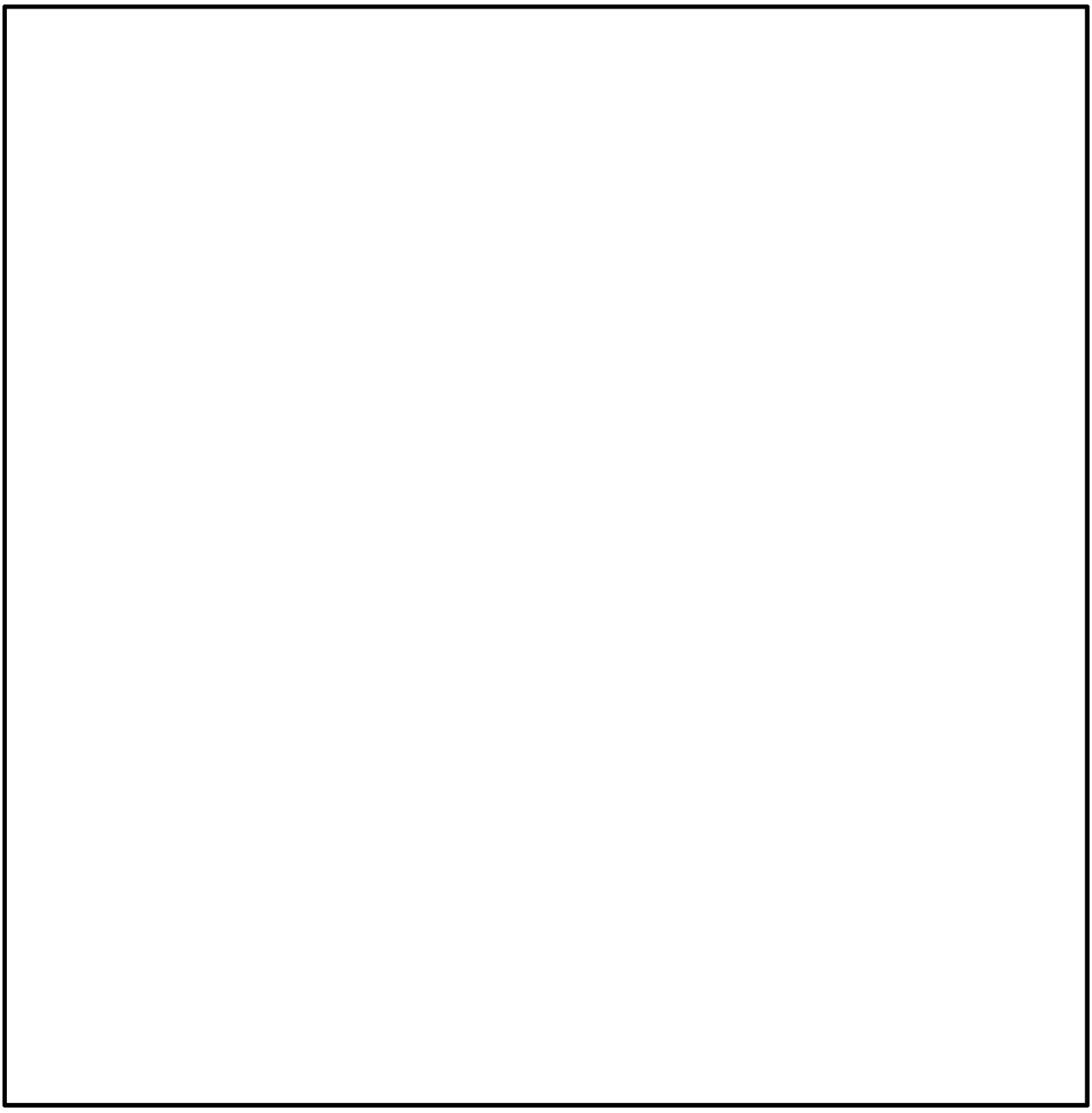

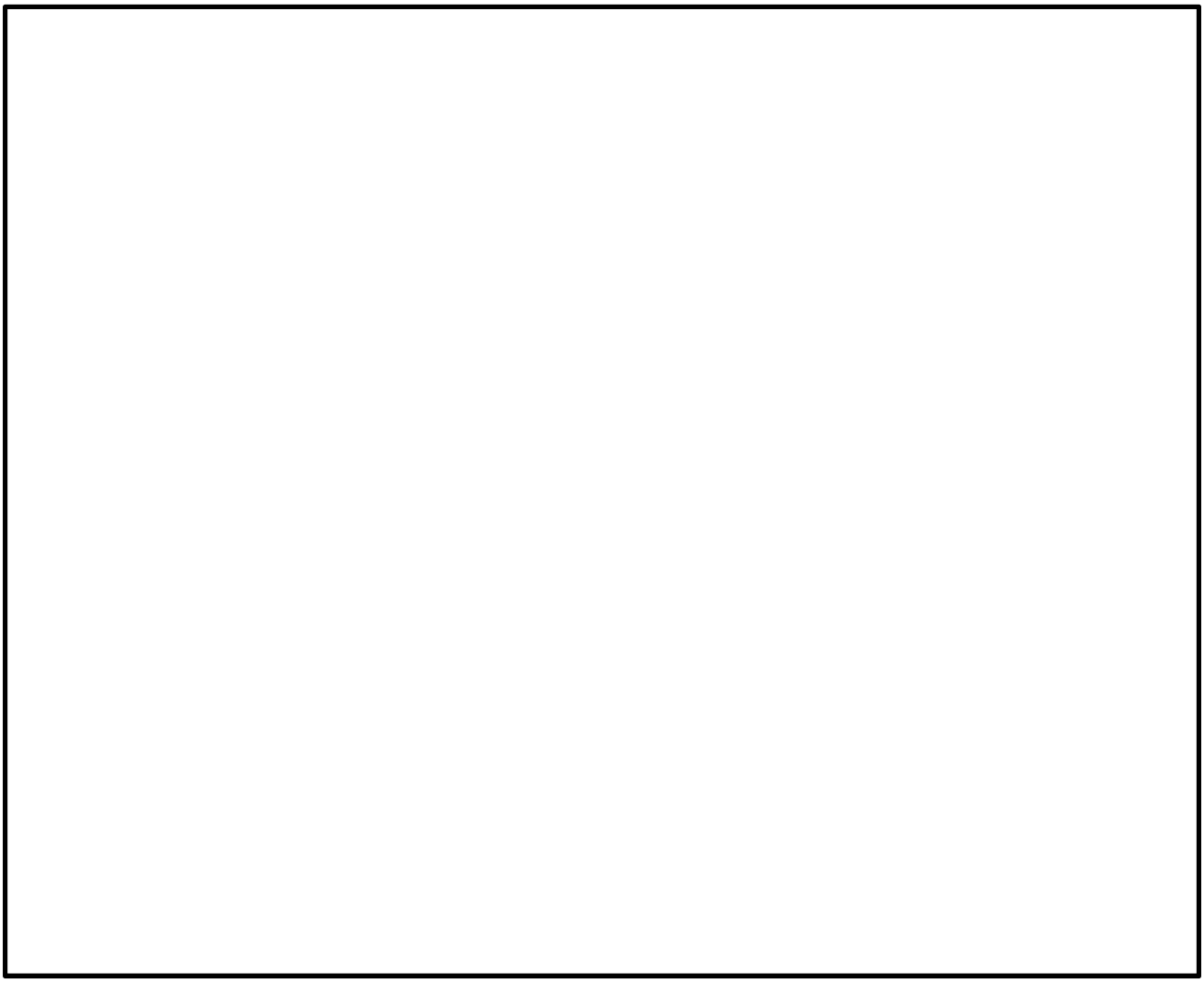

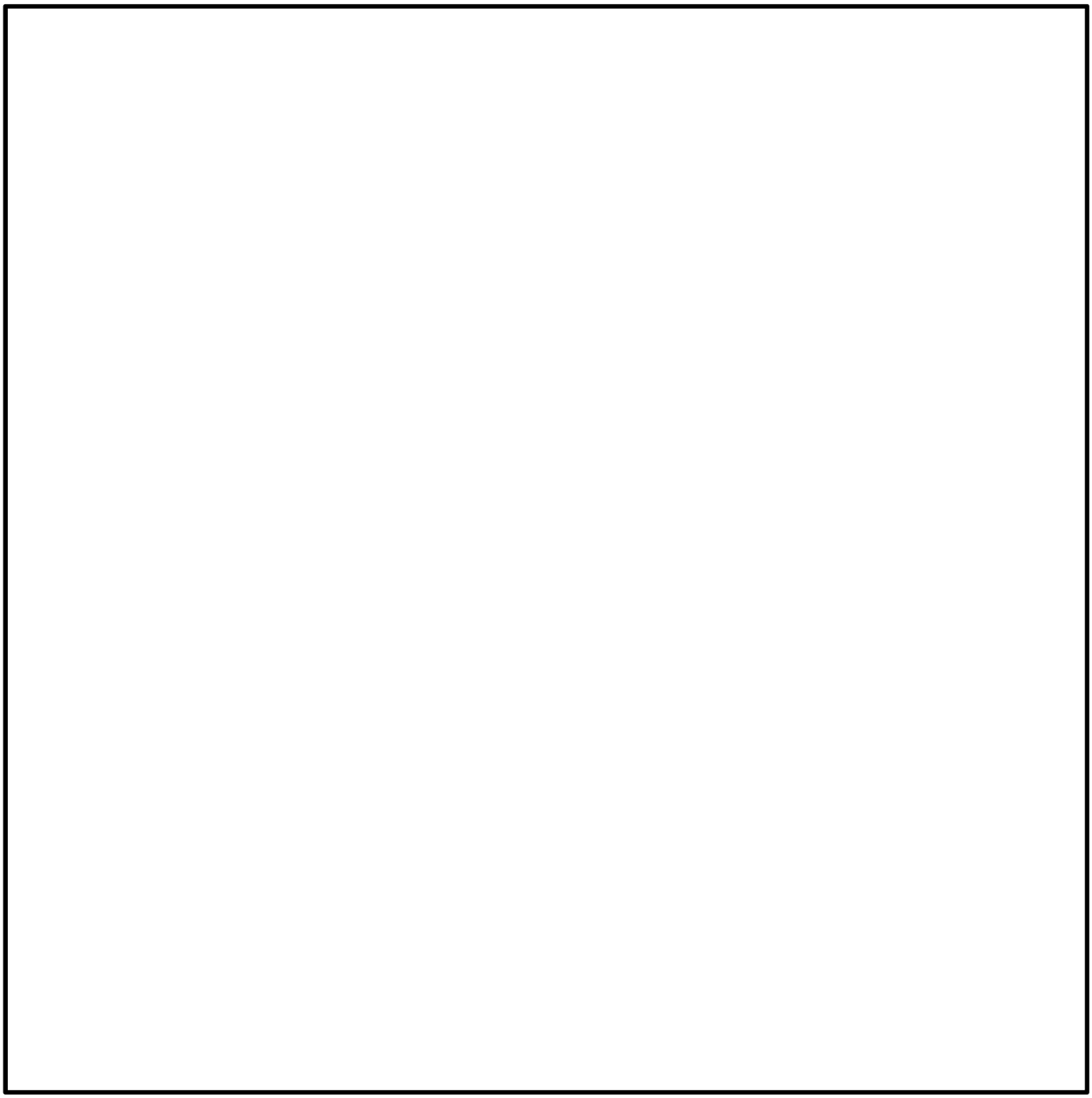

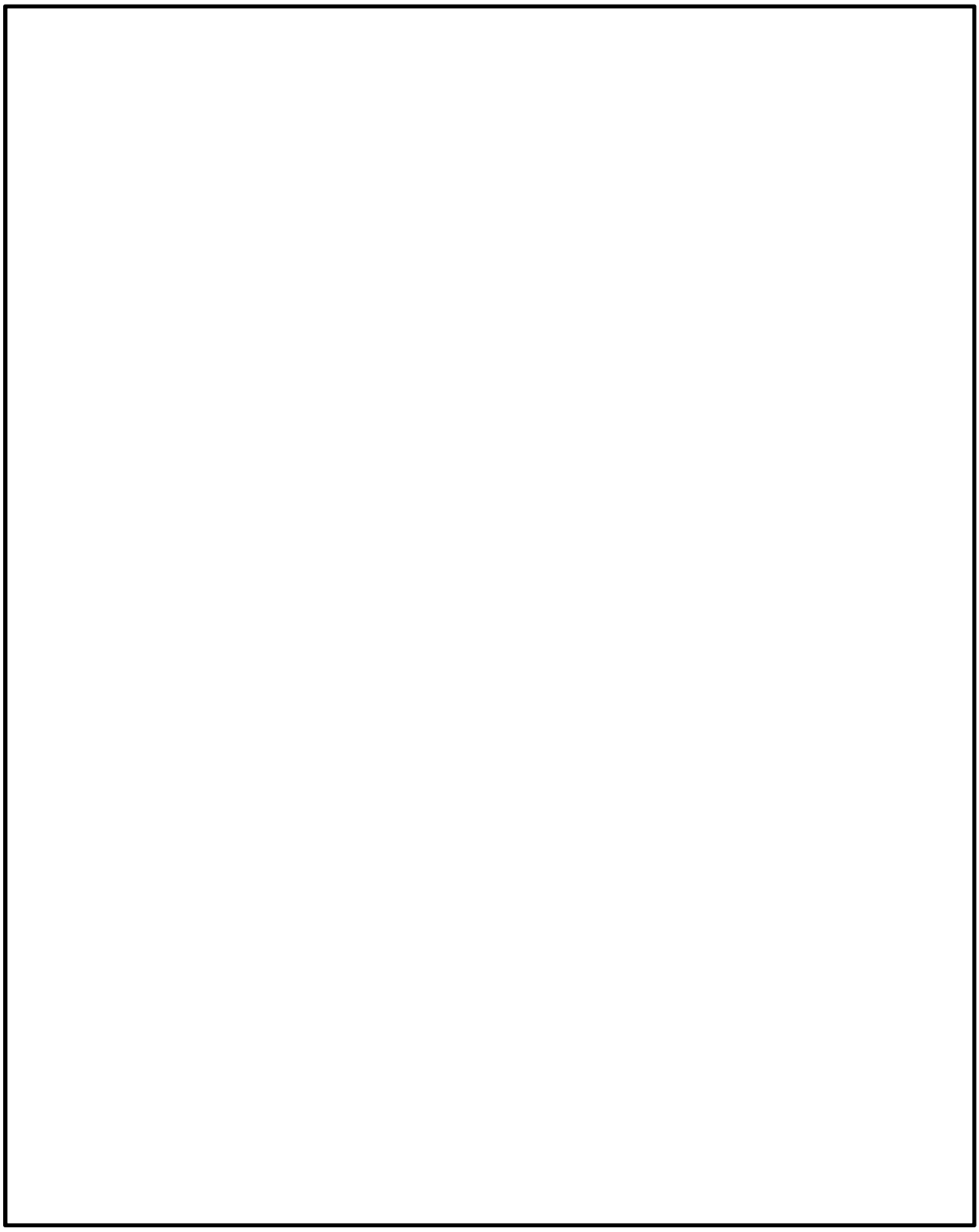

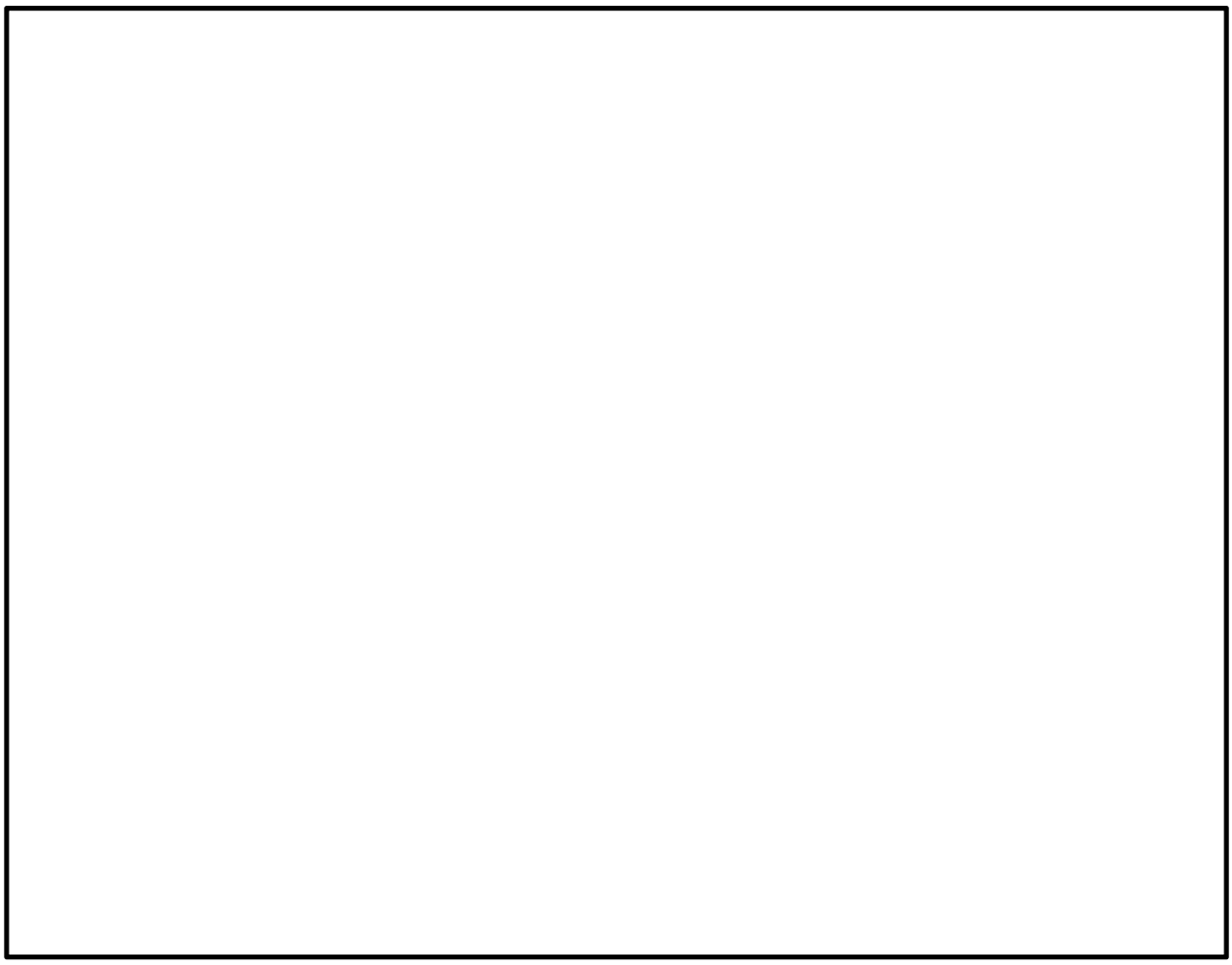

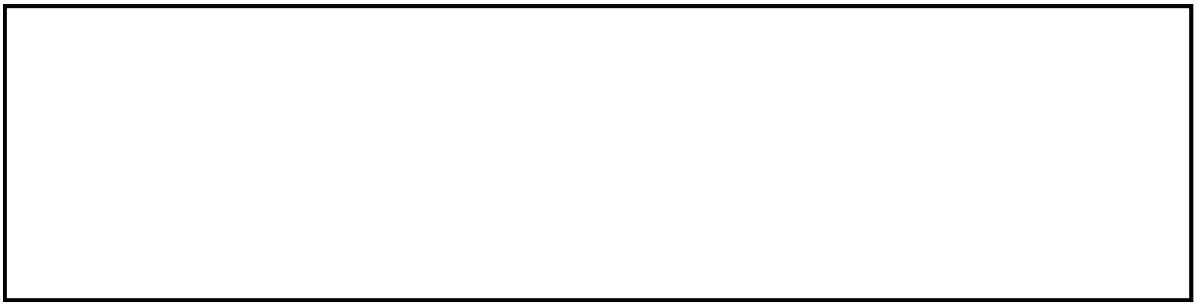

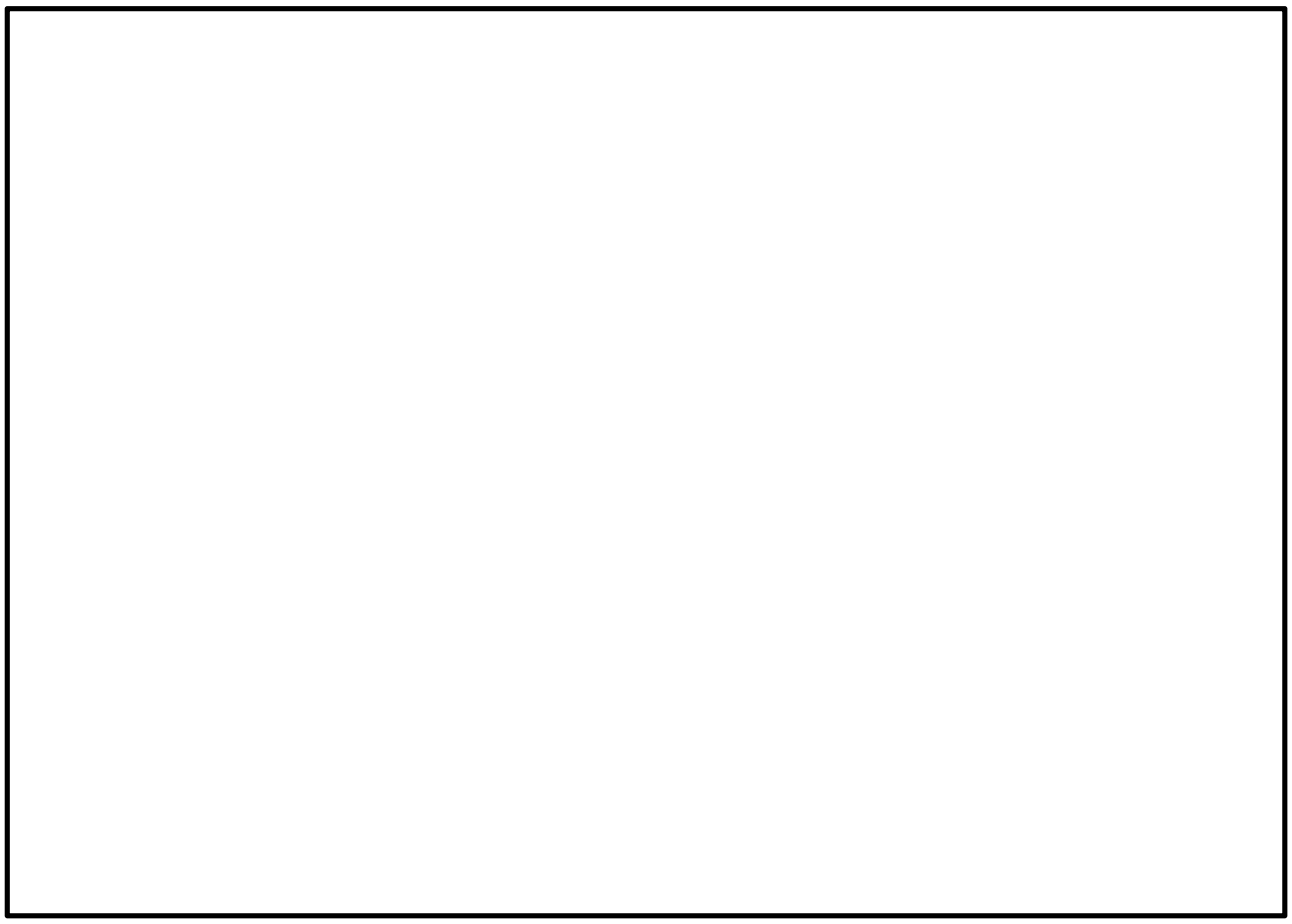

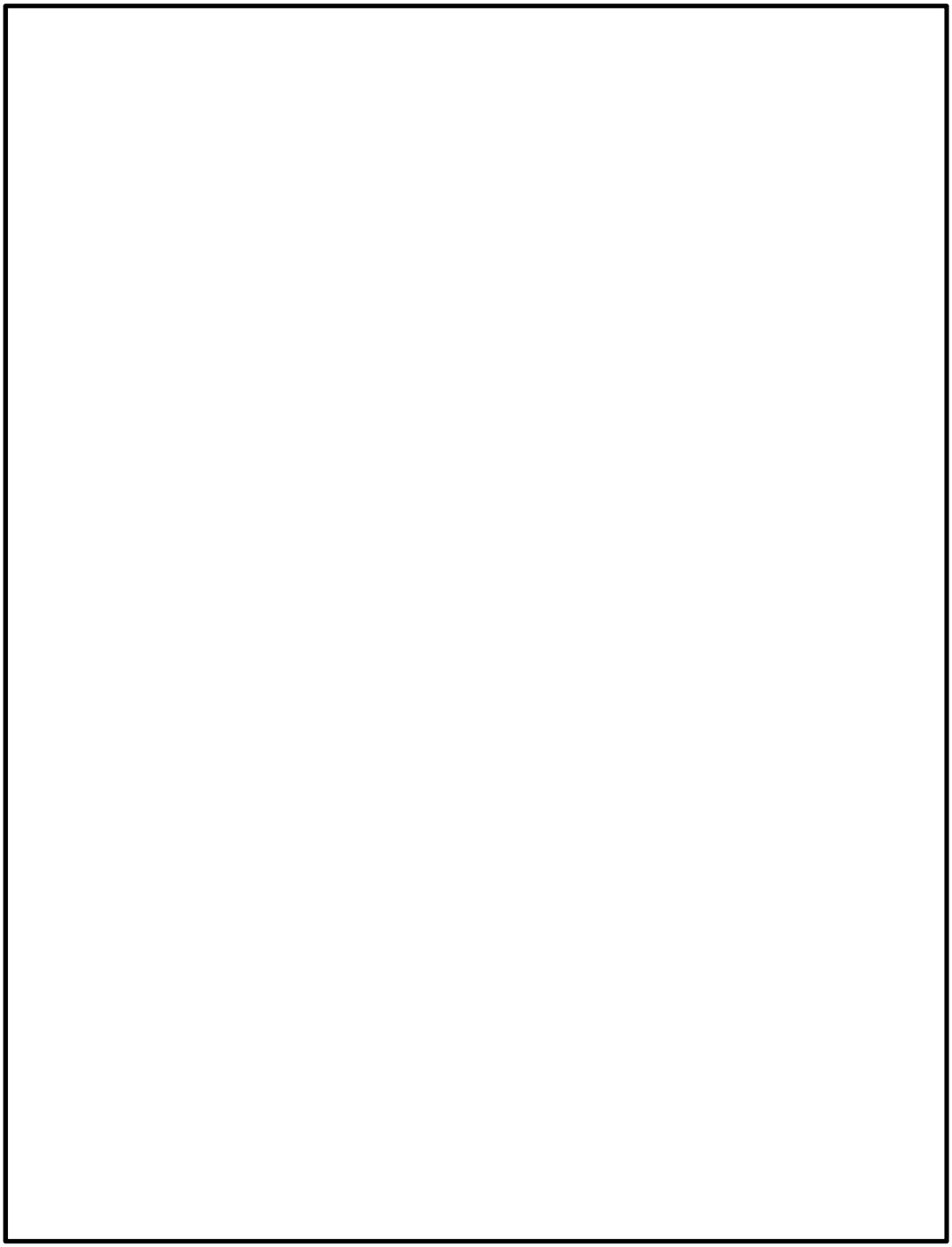

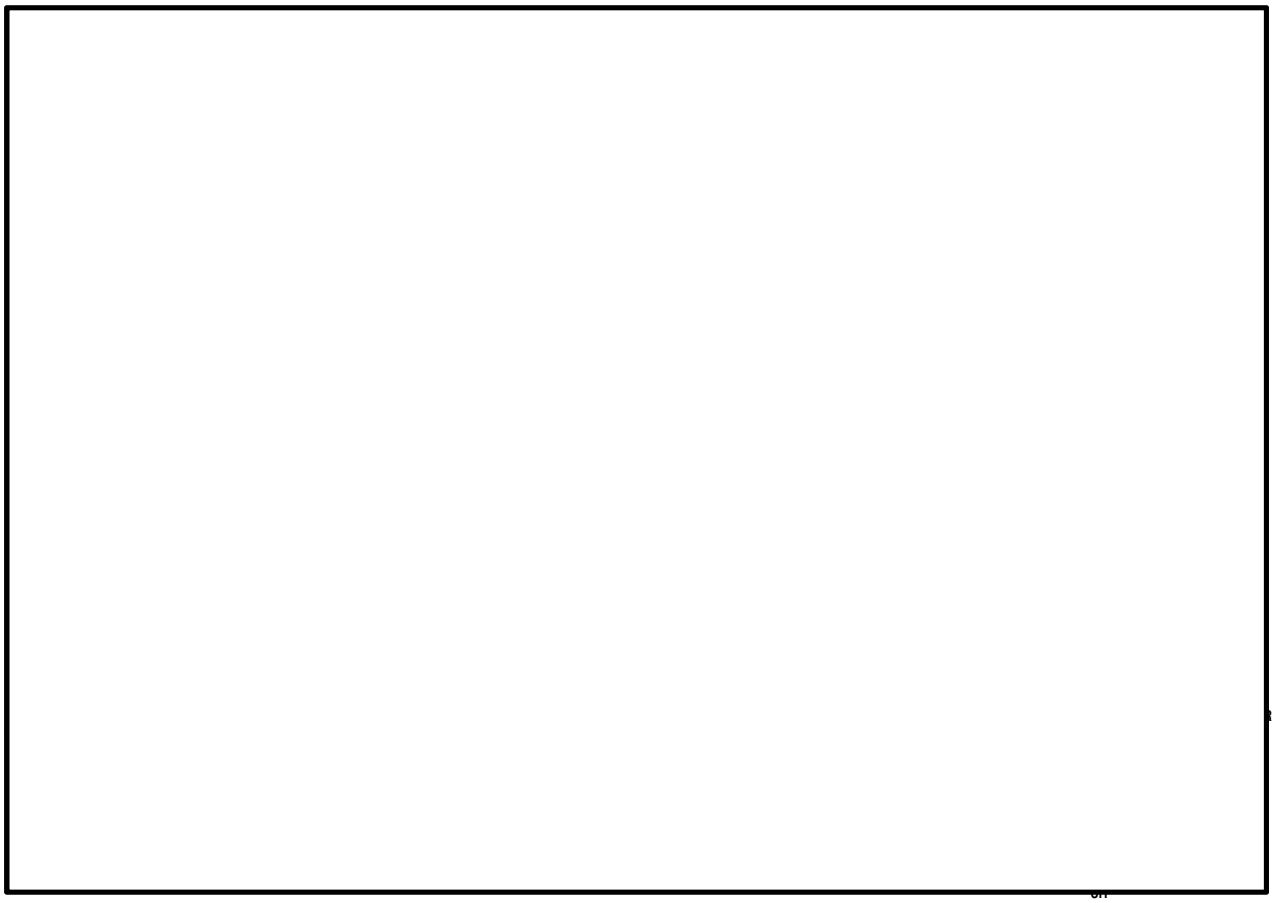

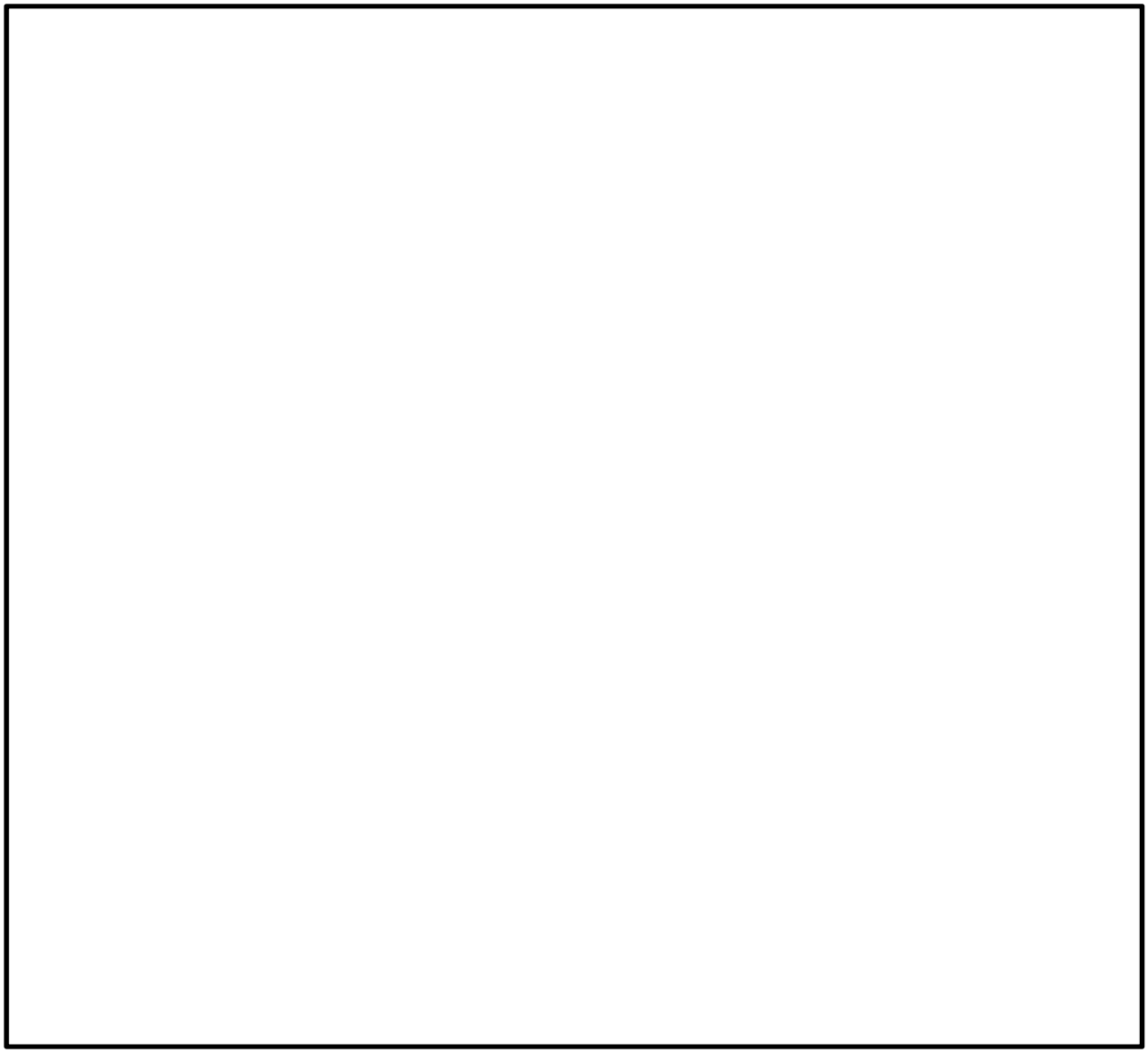

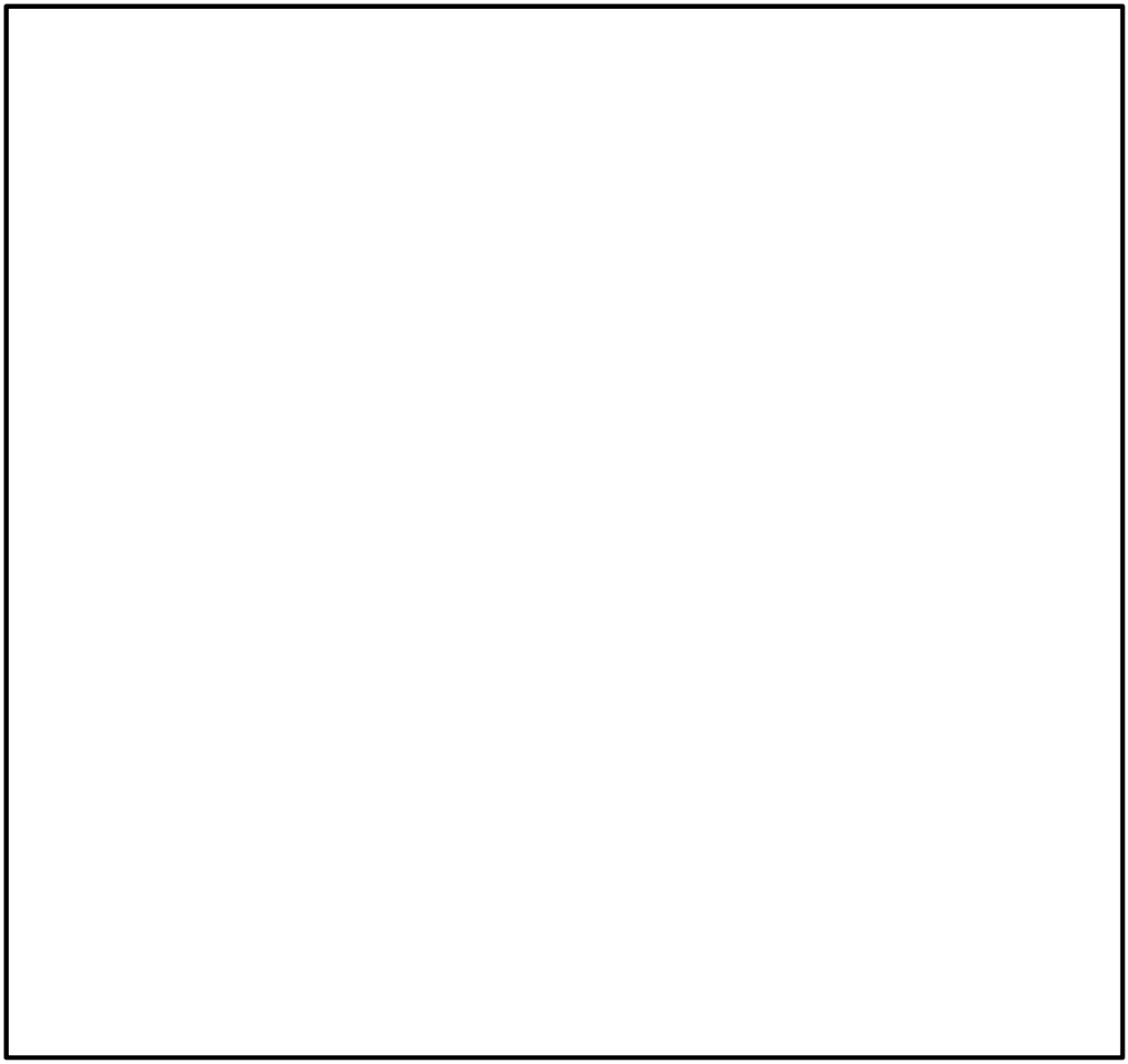

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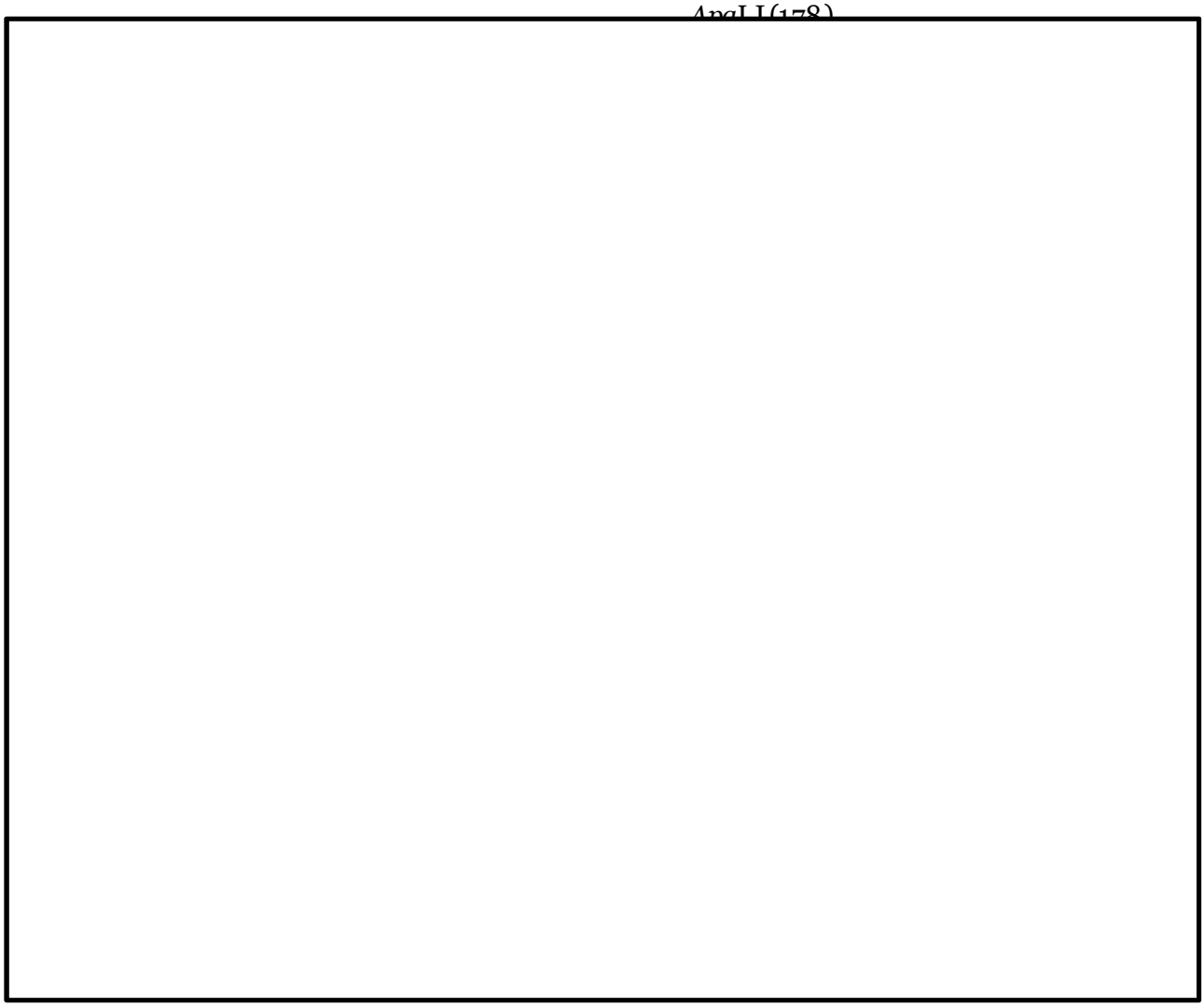
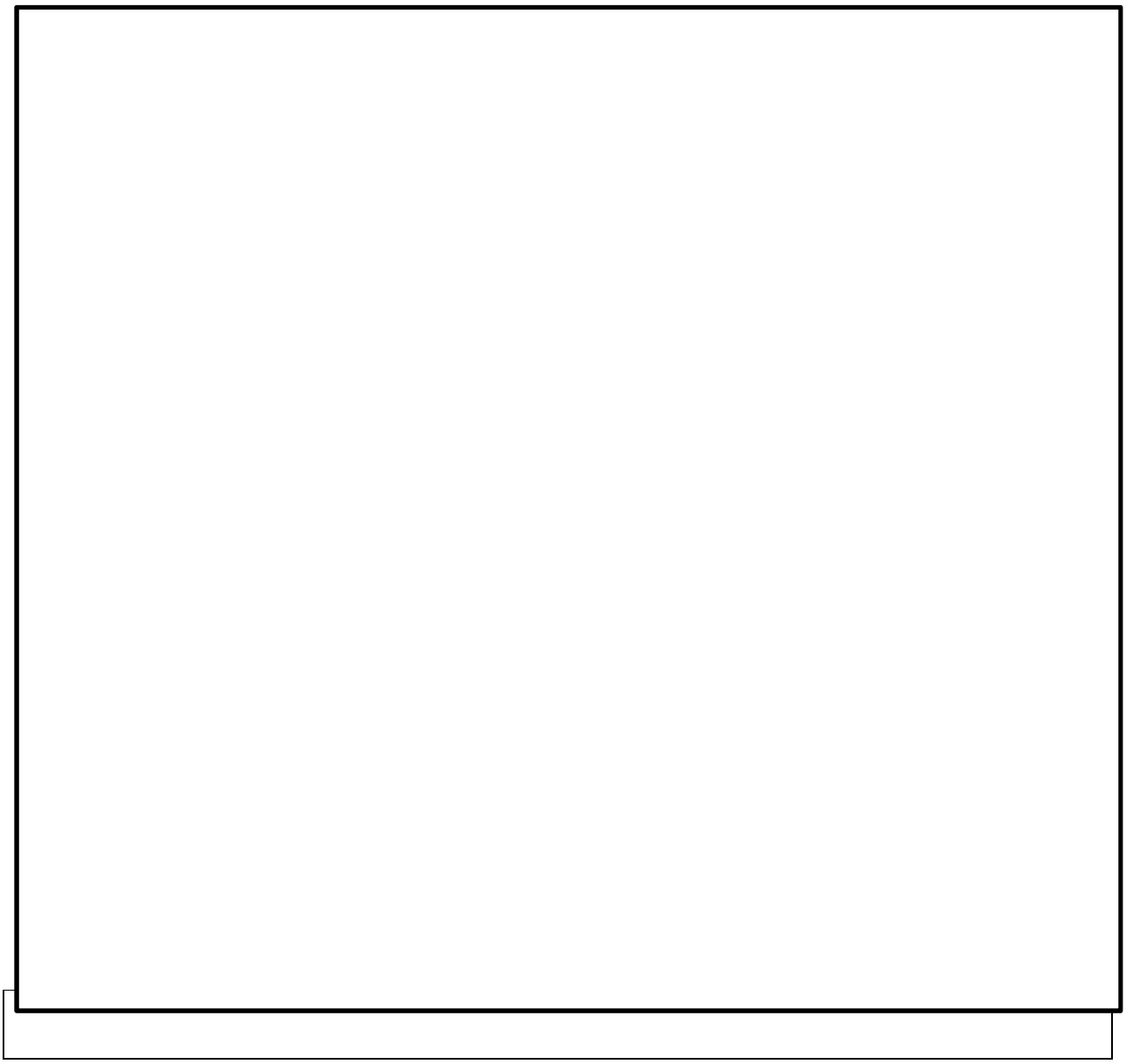
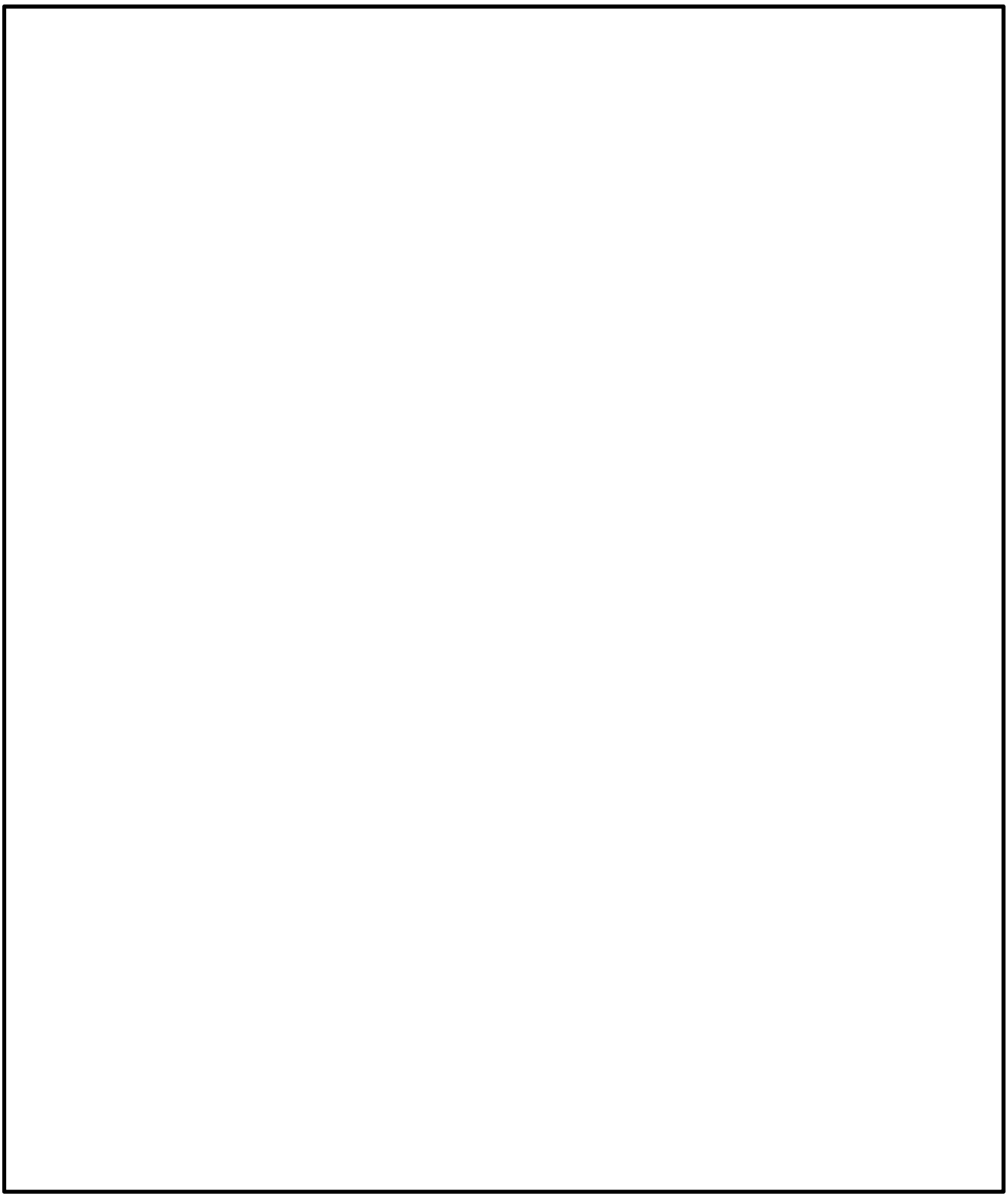
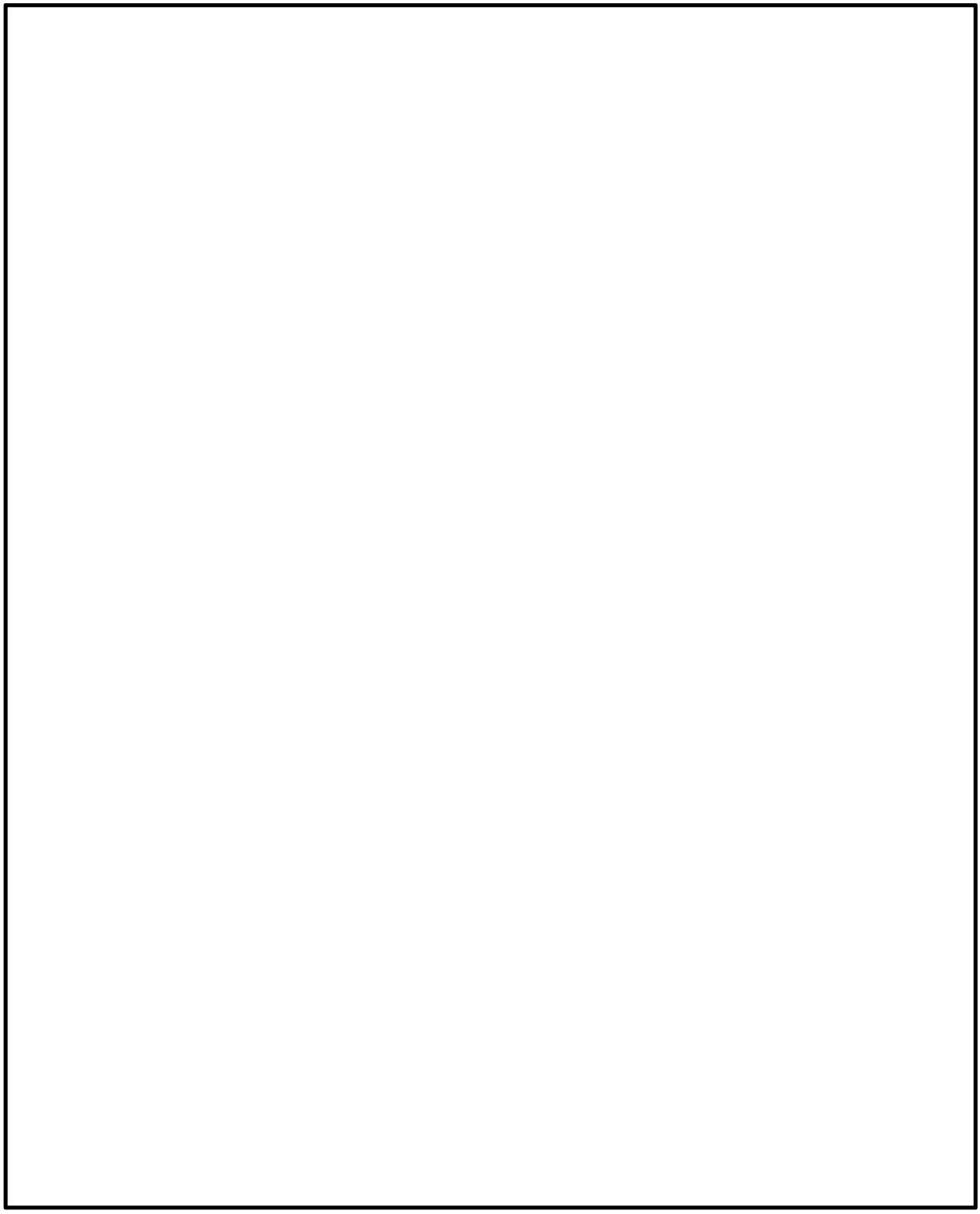
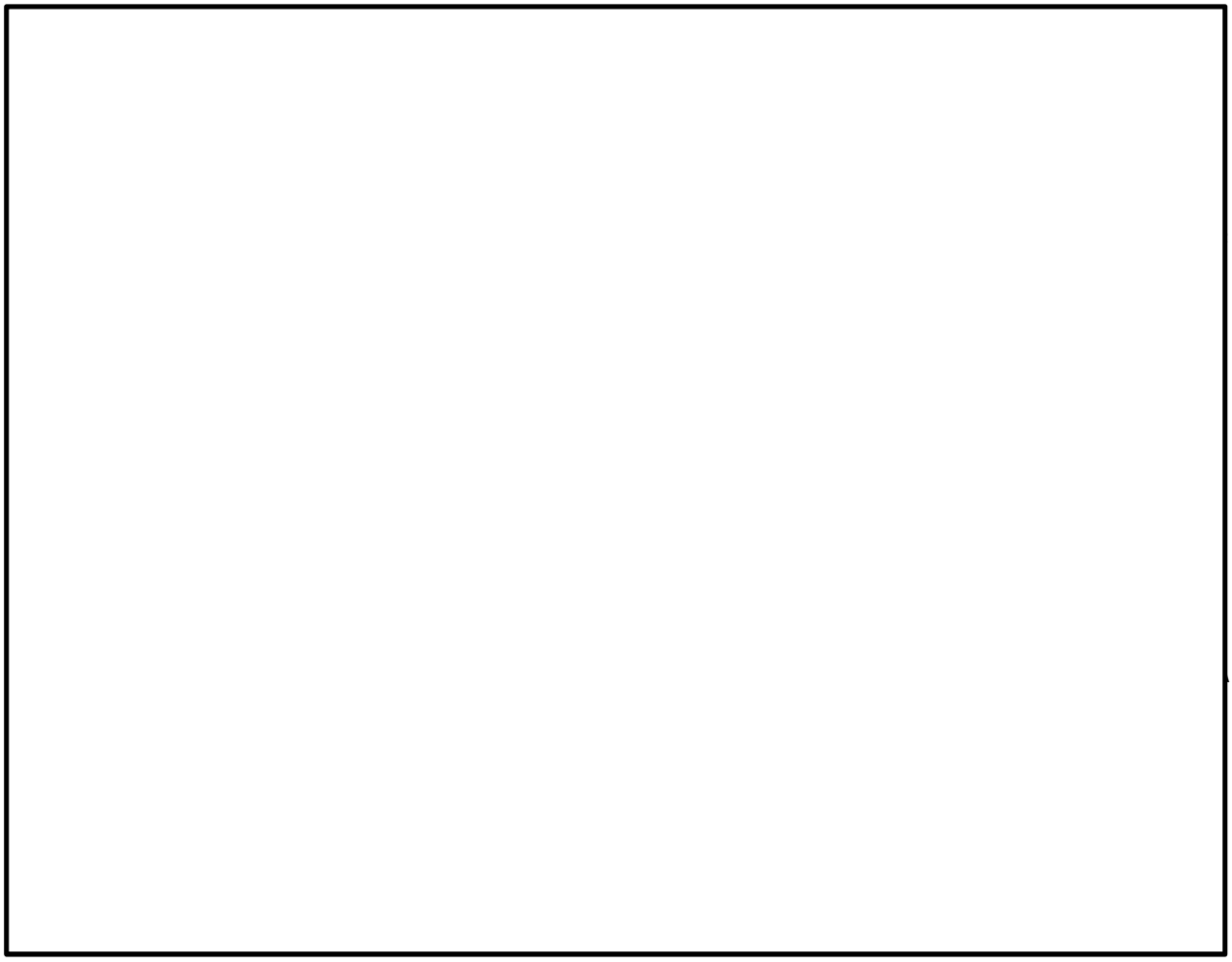


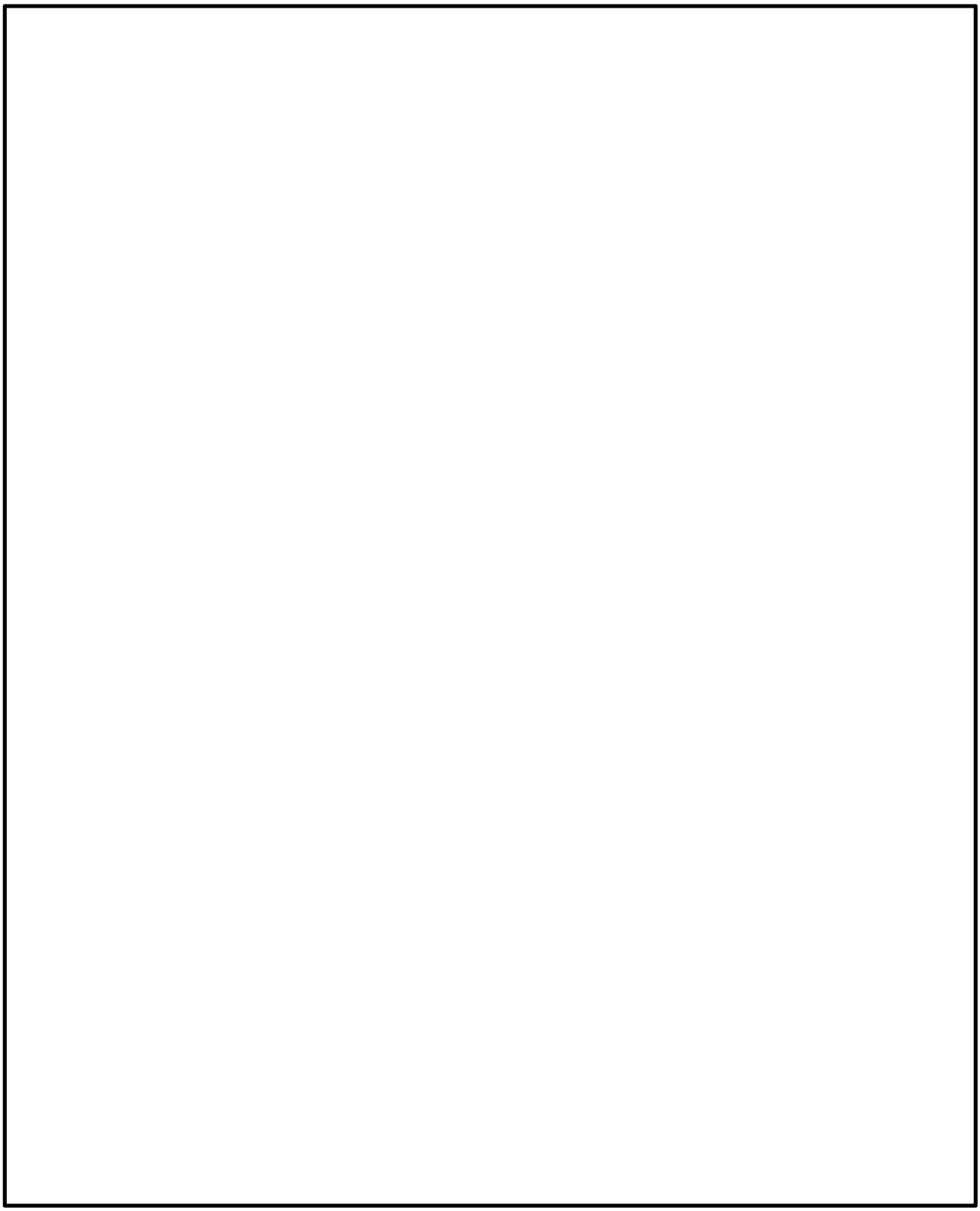
Figure 18. Sequence of the 633 bp DNA fragment containing the *S. cerevisiae FBA1* promoter (primer sequences highlighted in bold).

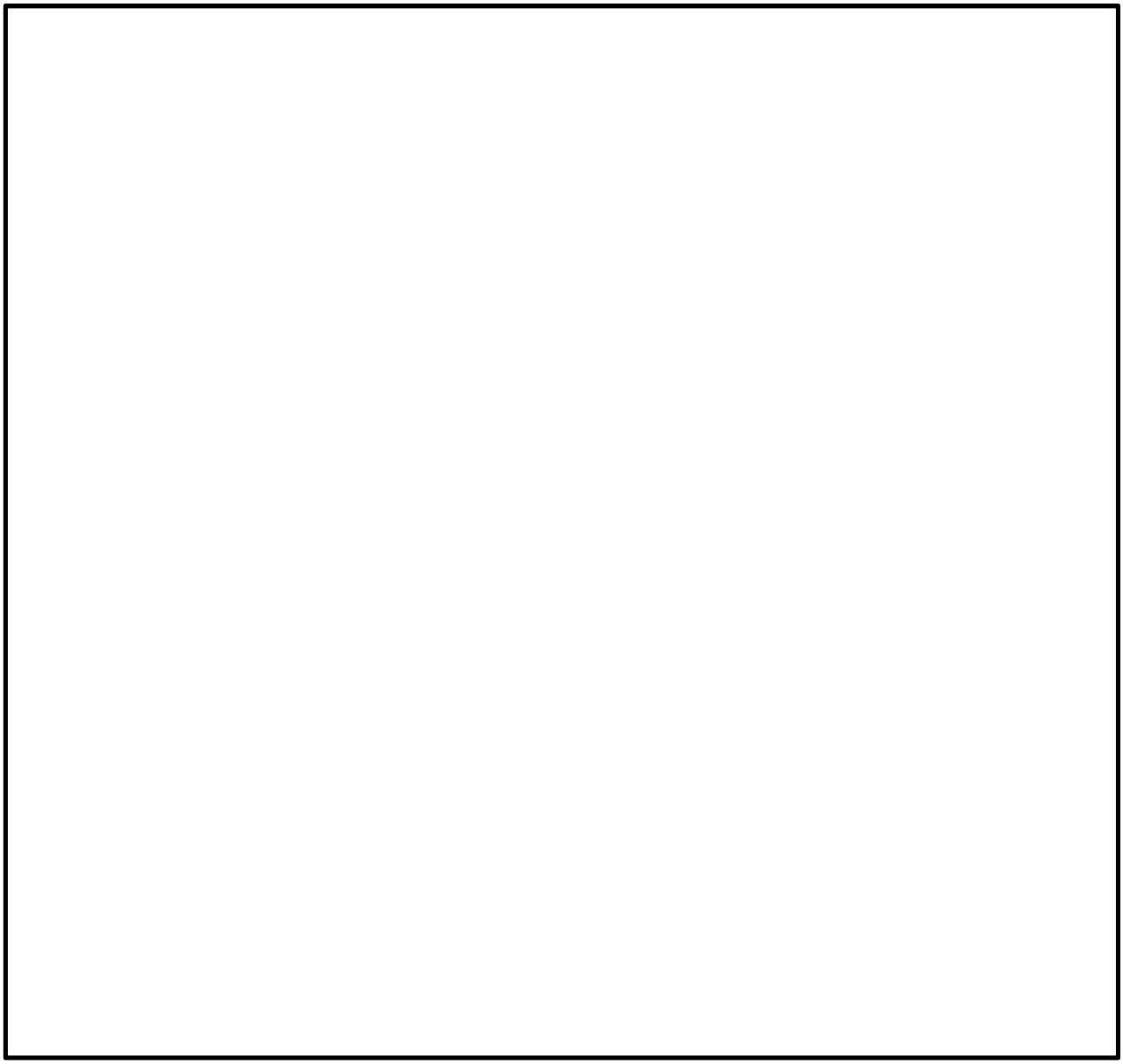


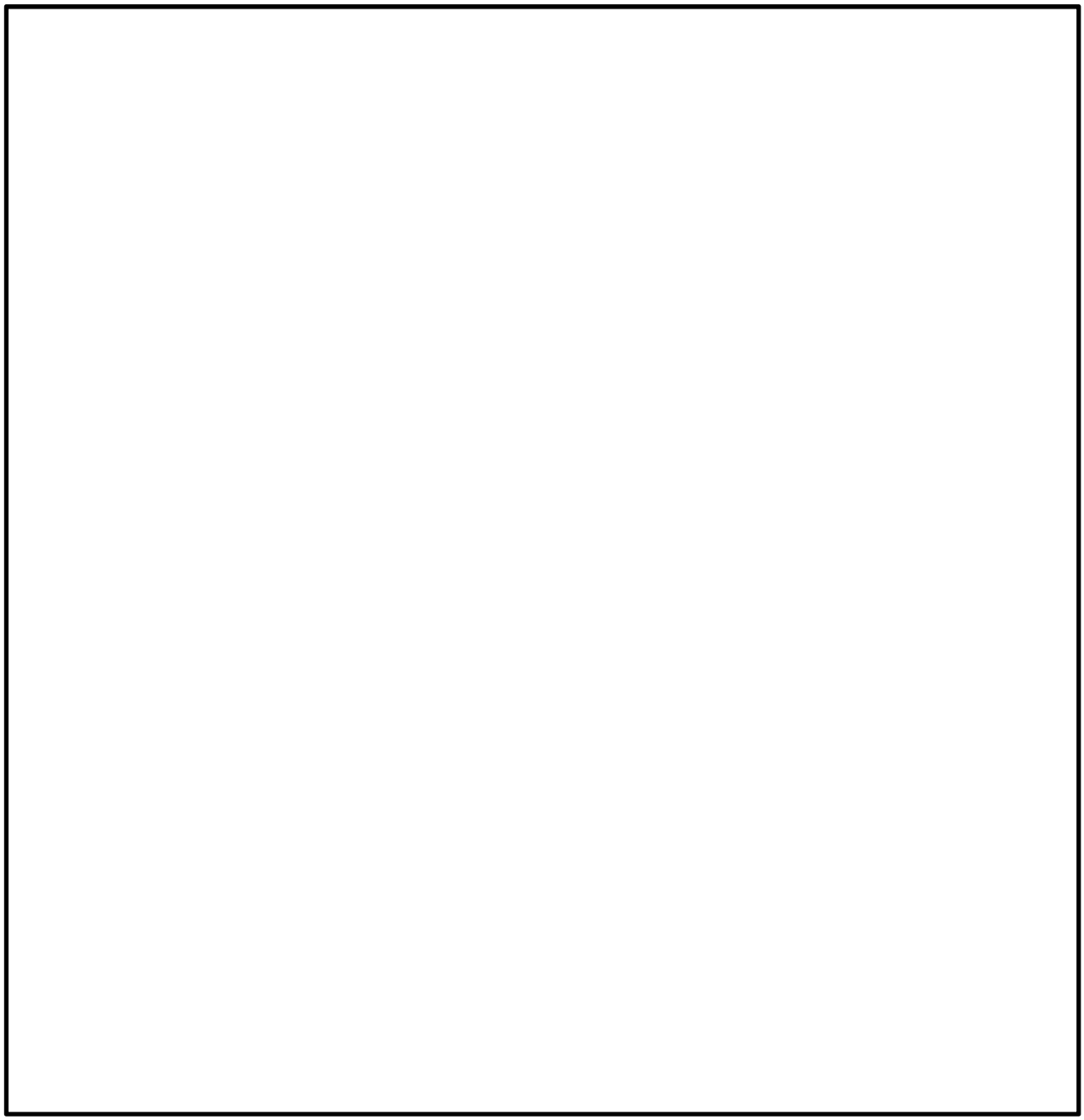


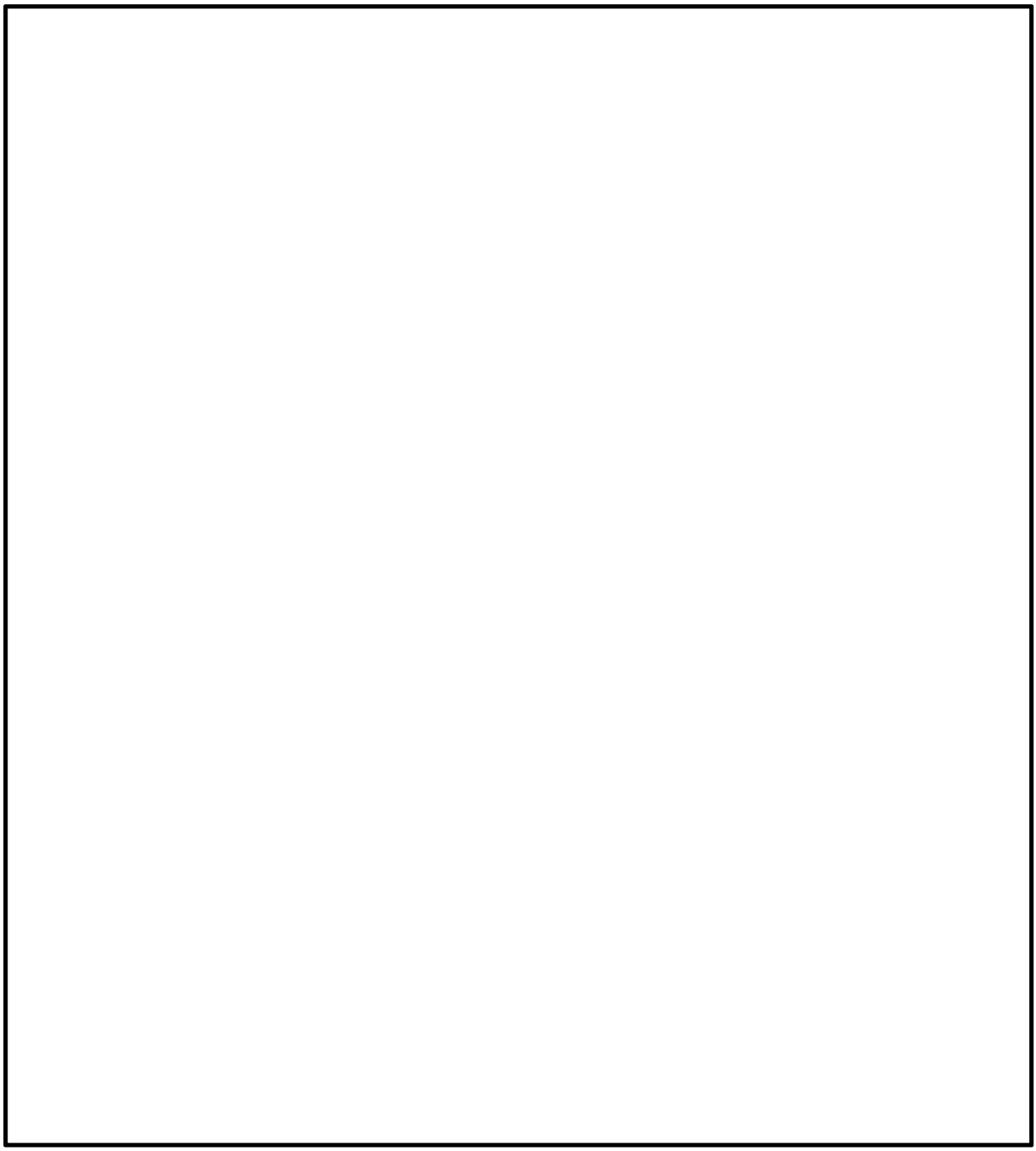


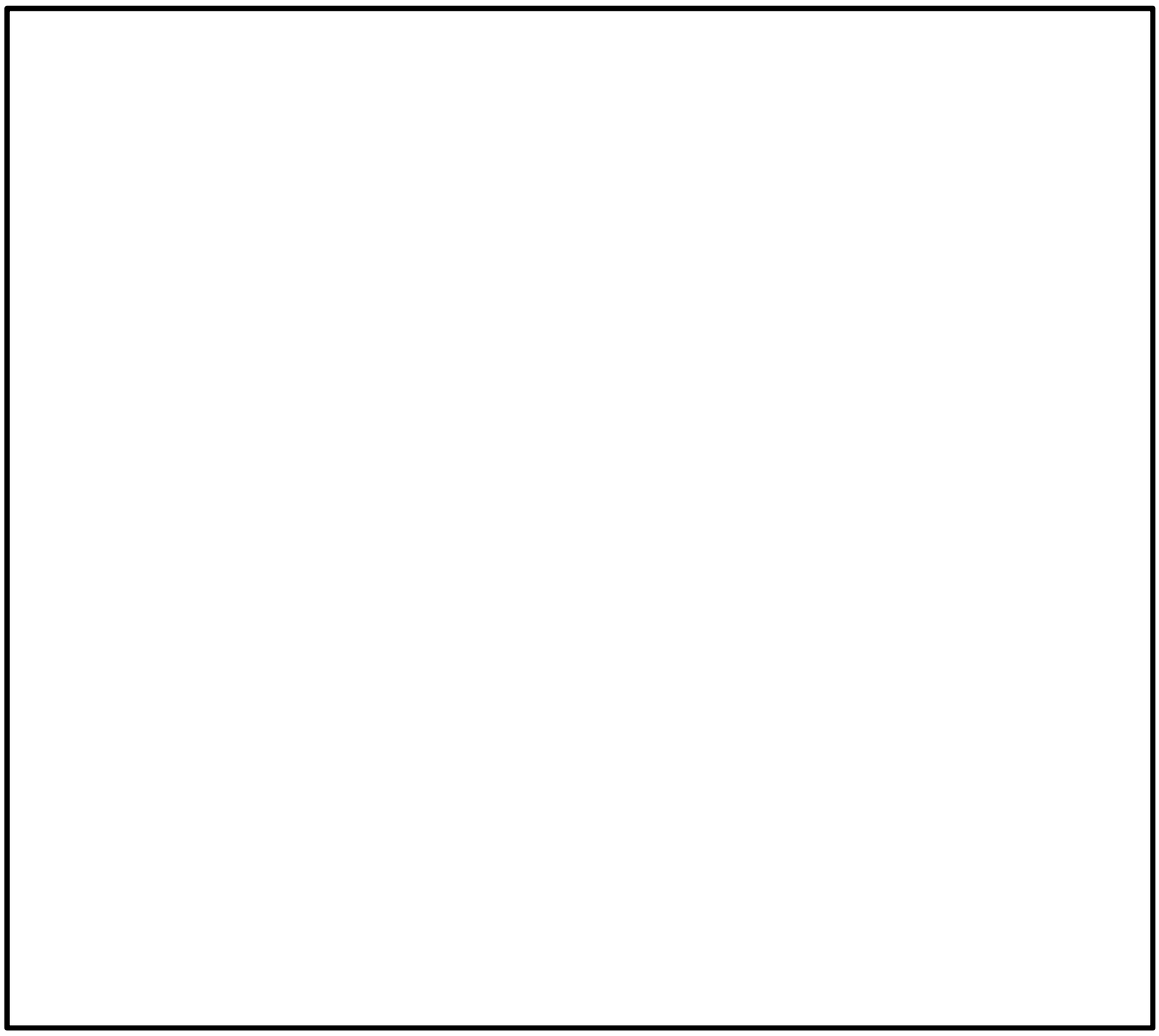


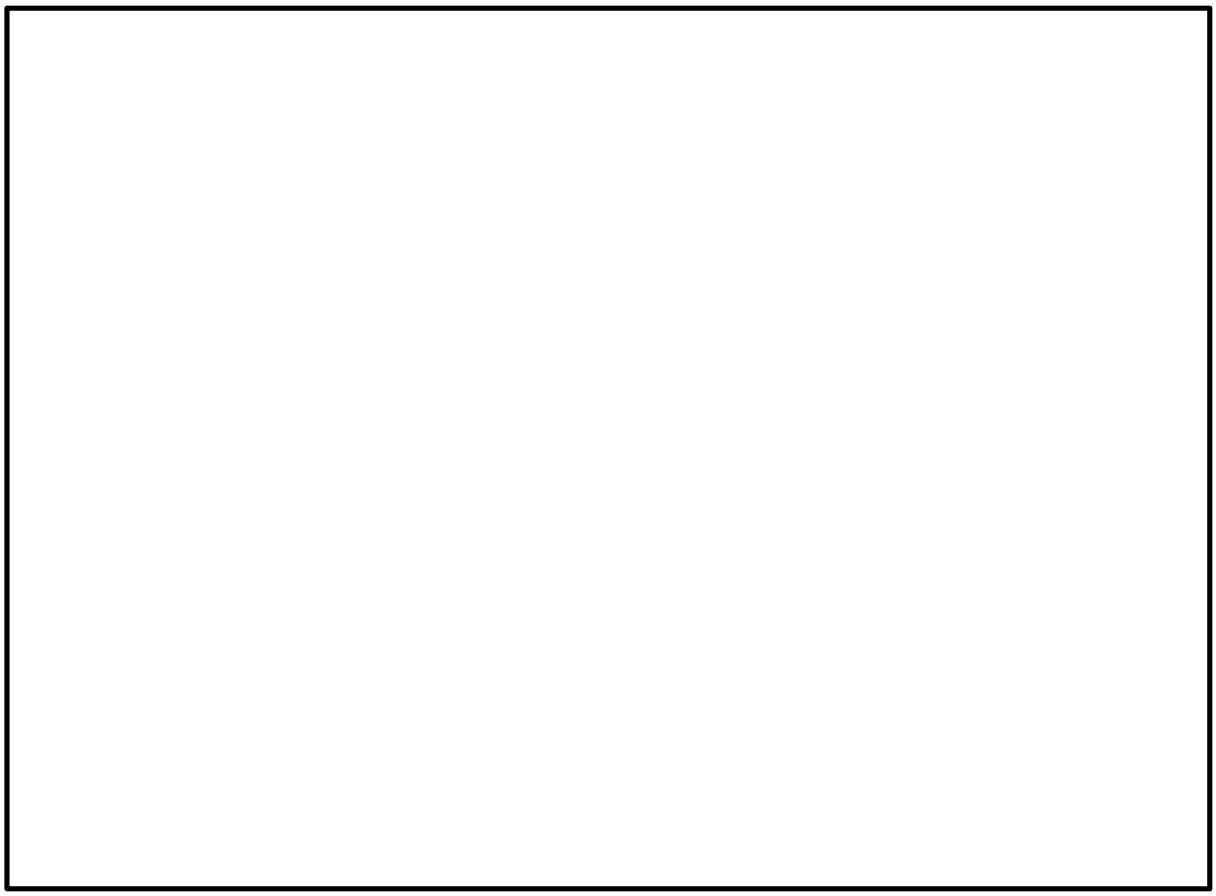


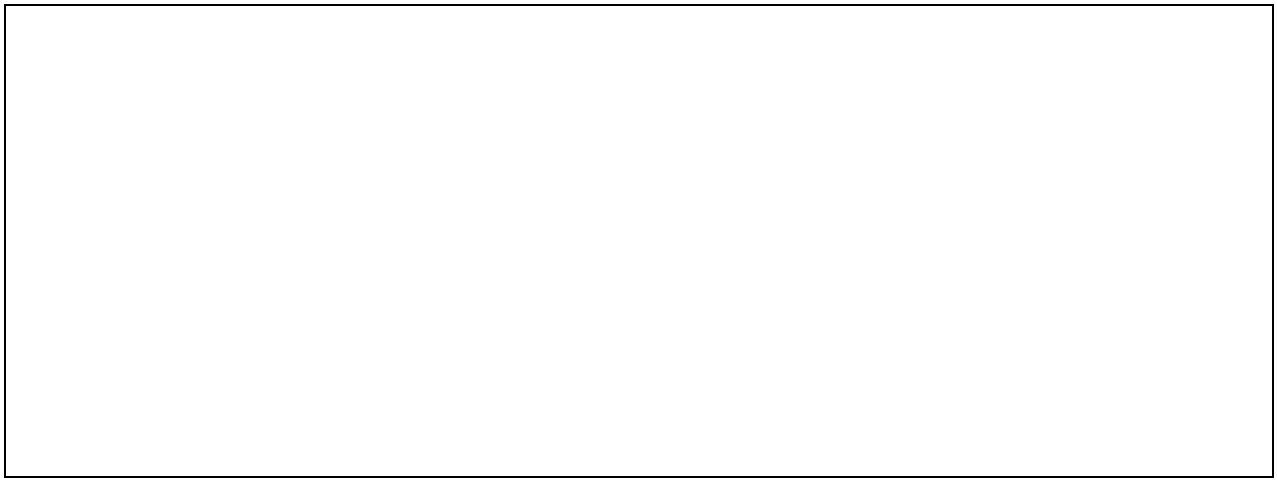


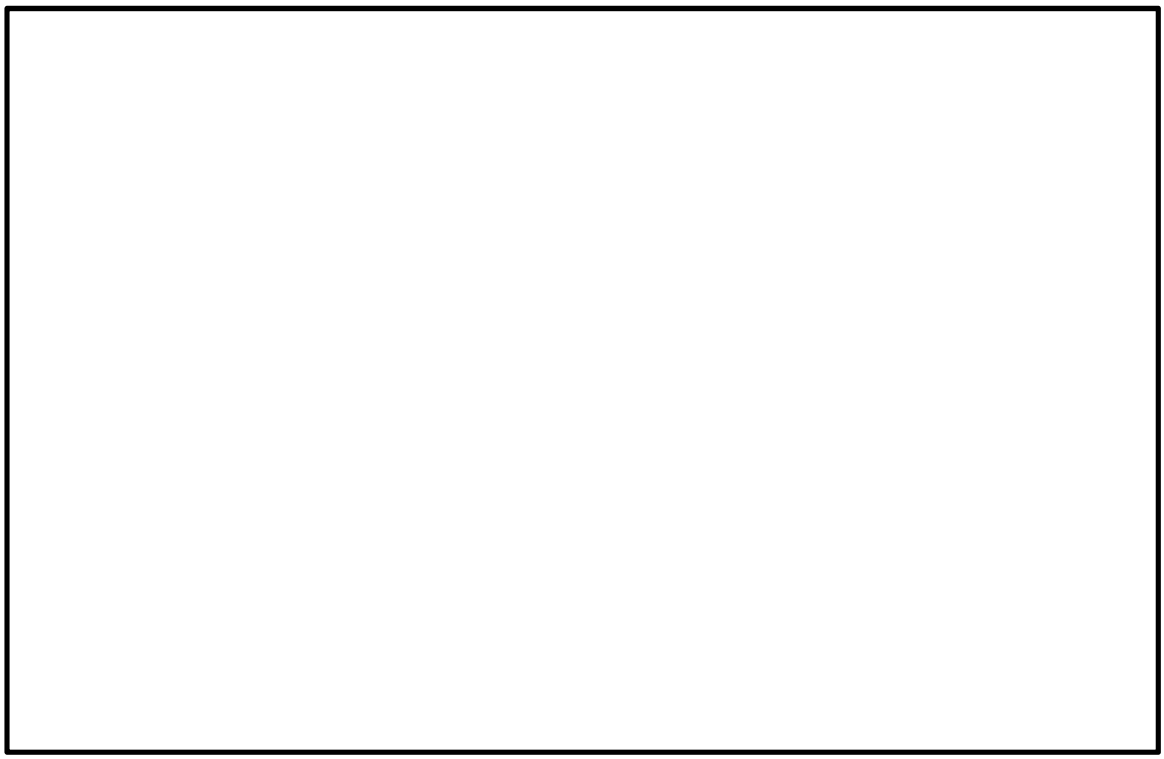


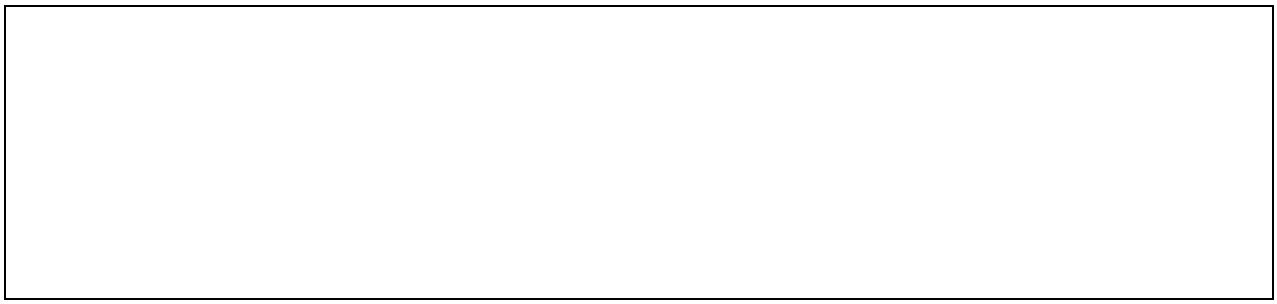


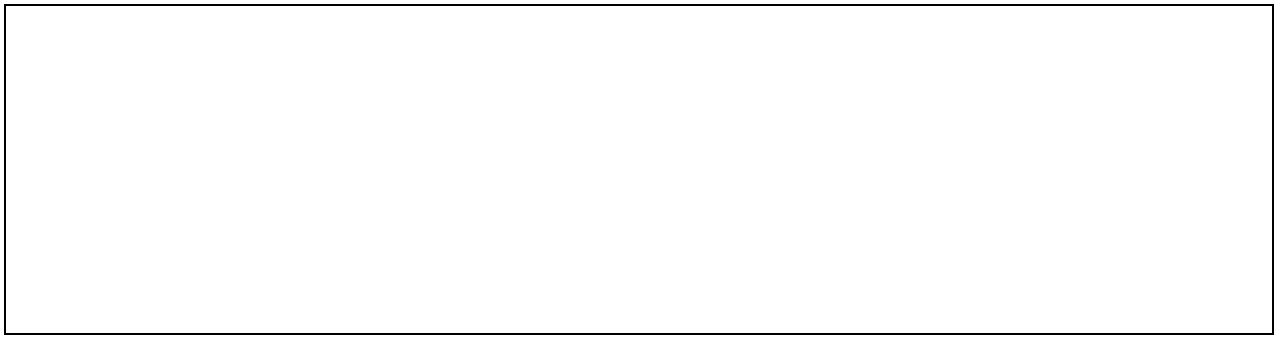


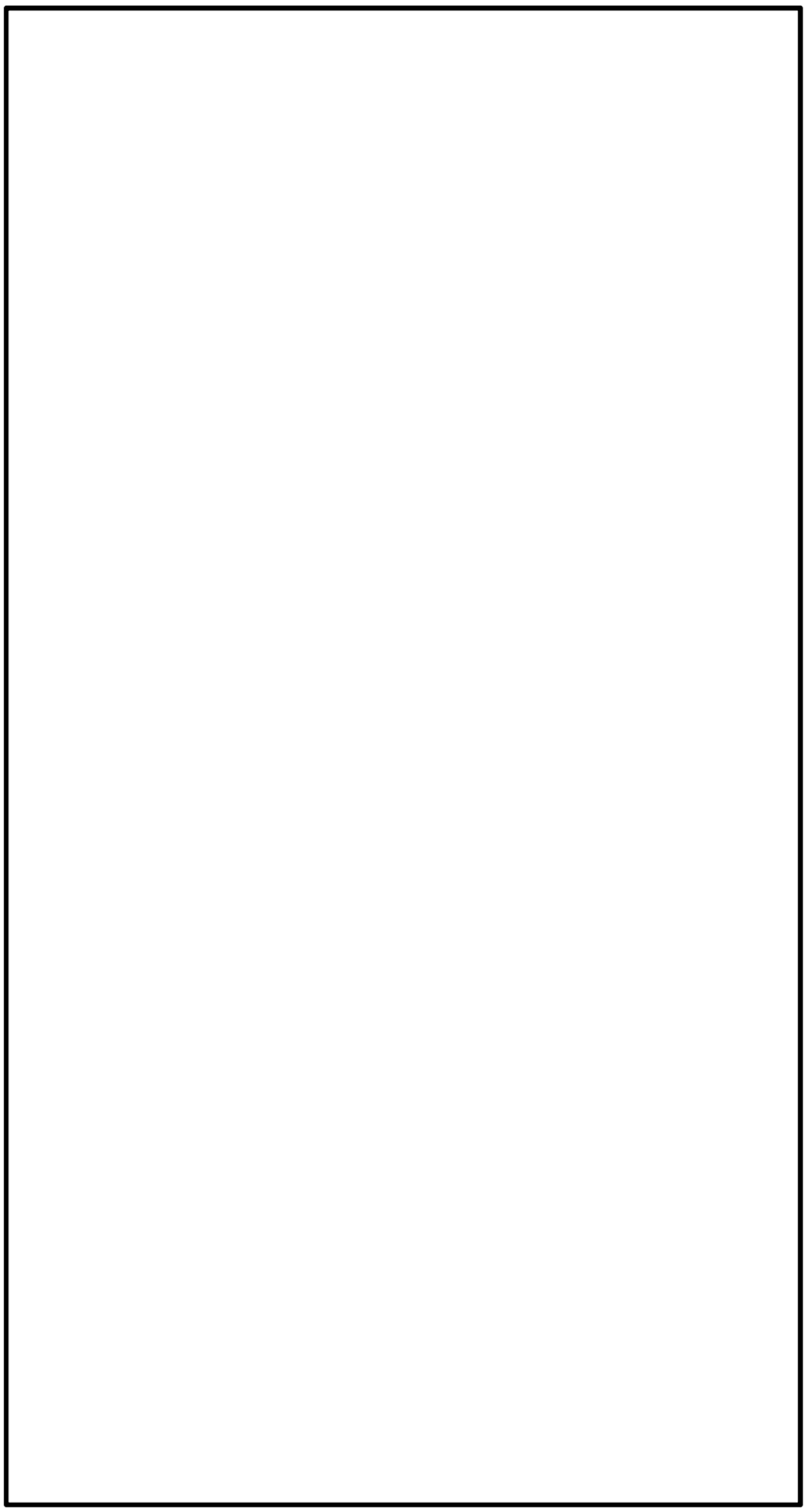


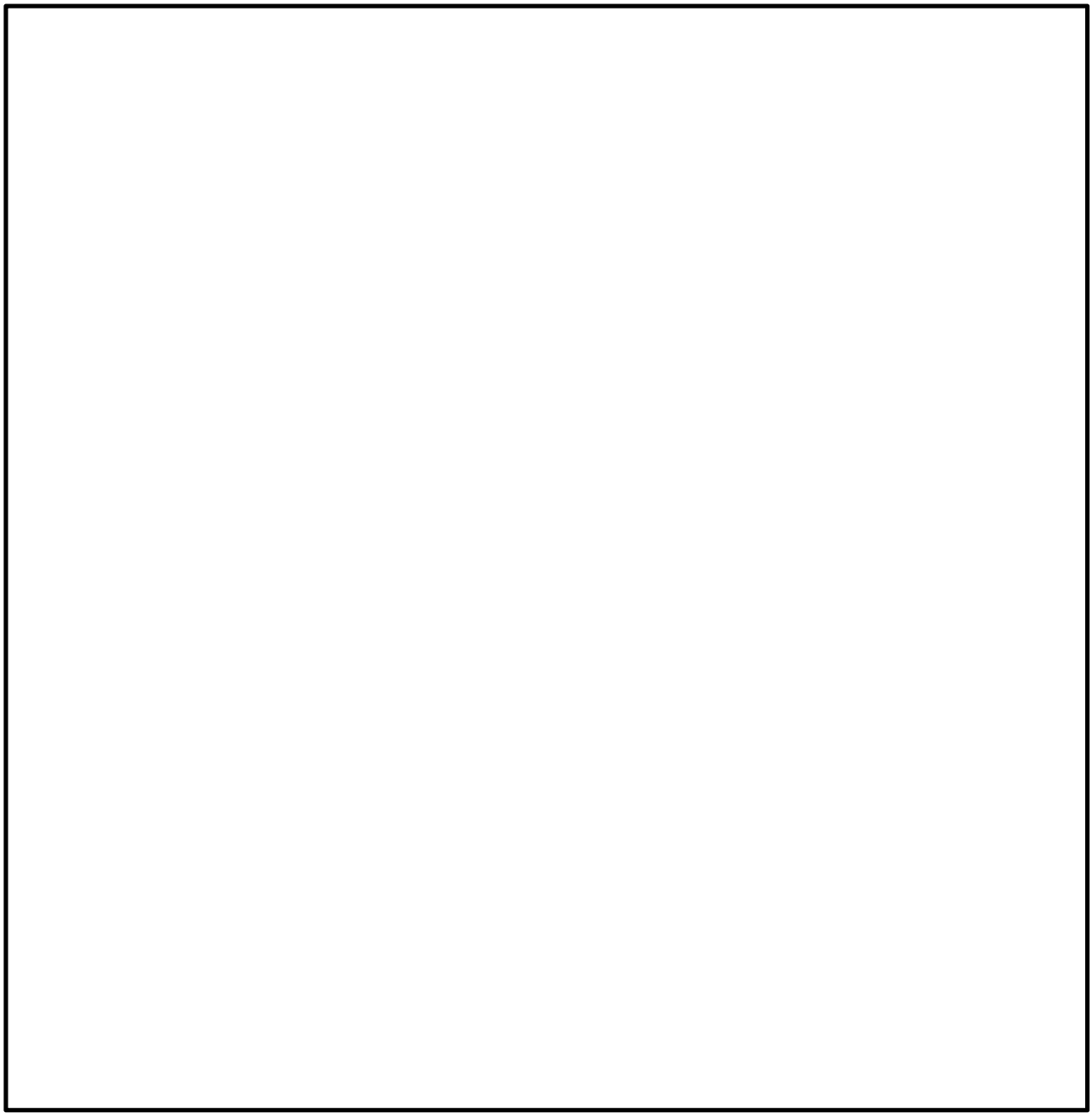


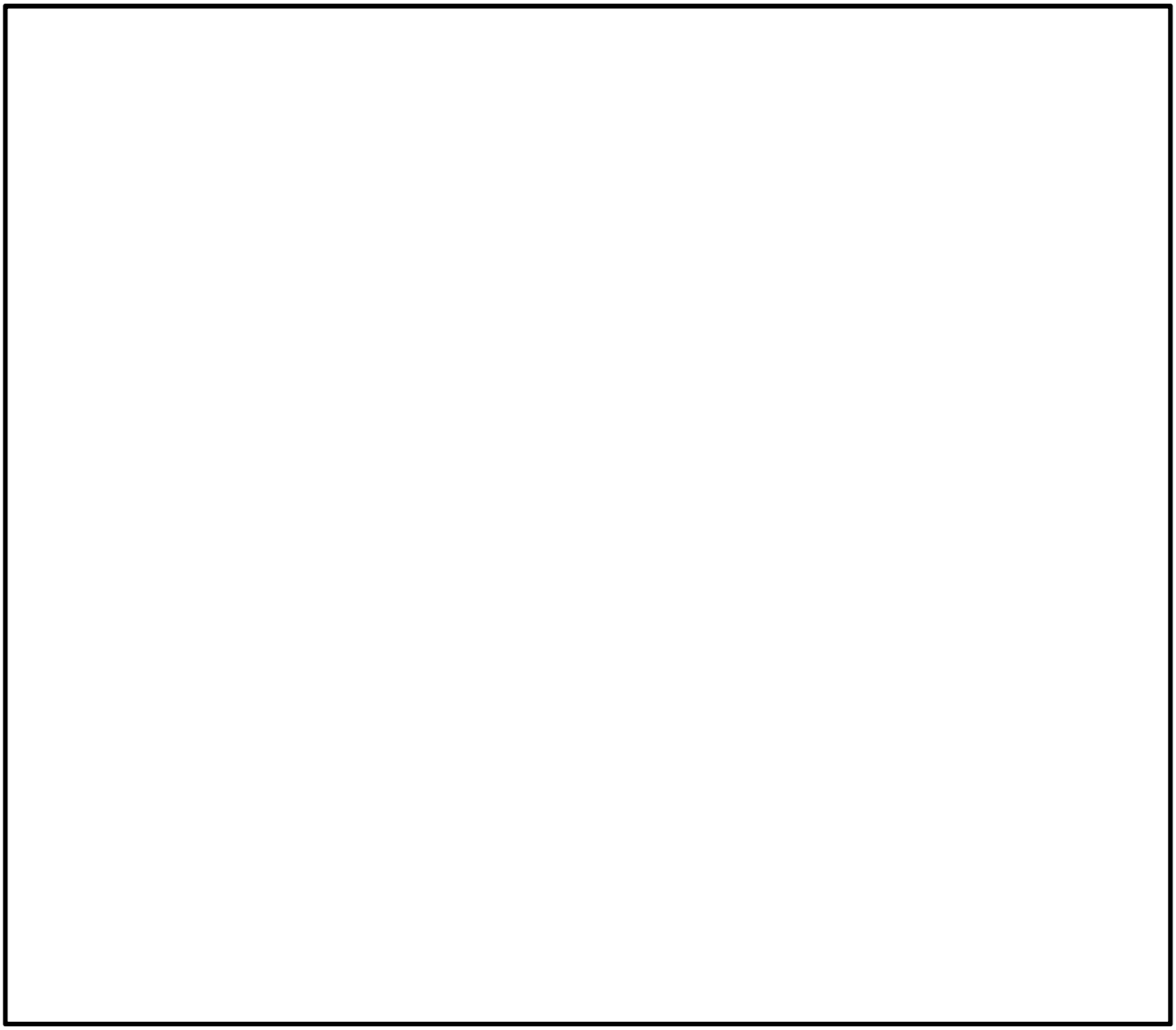


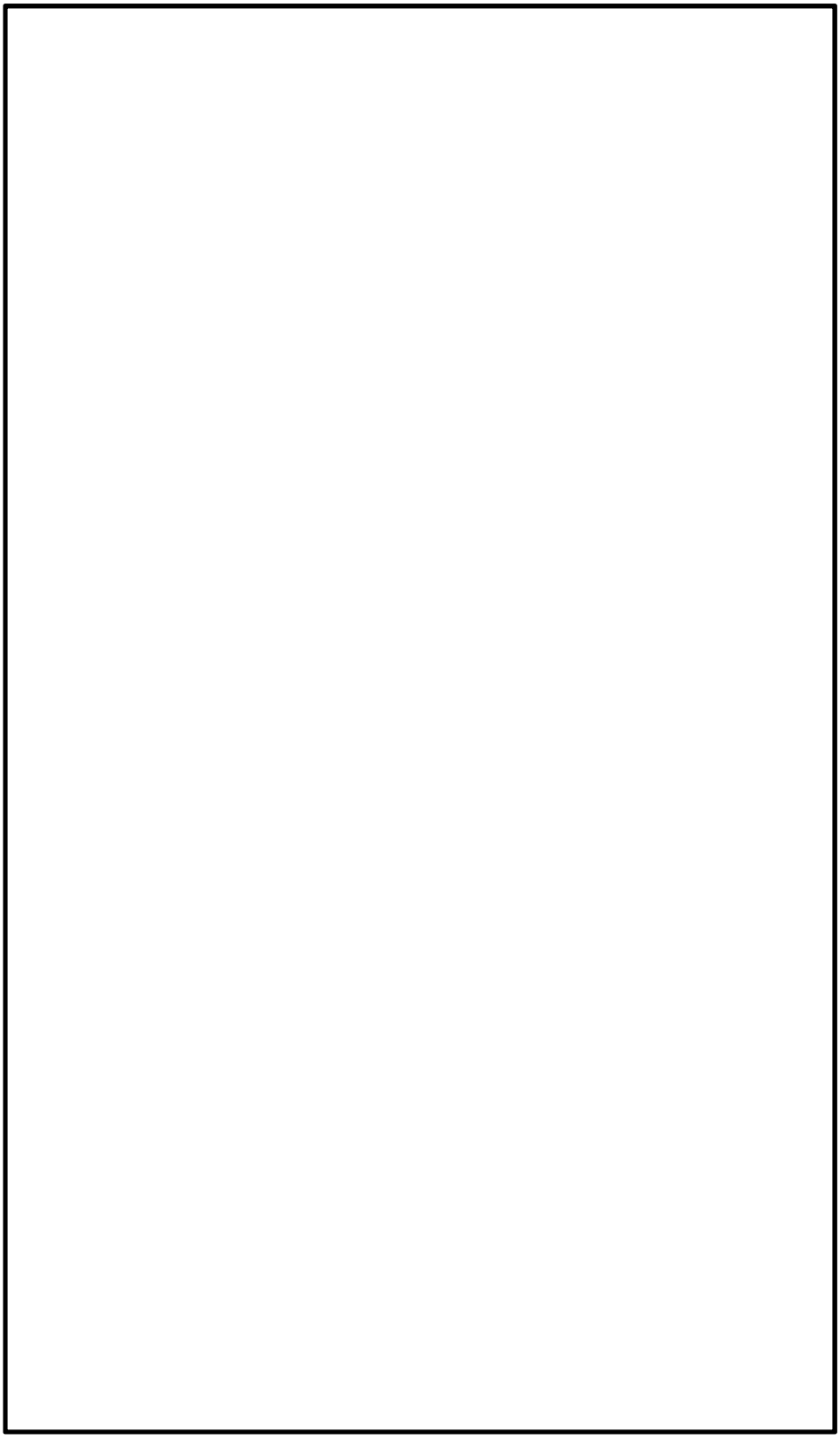


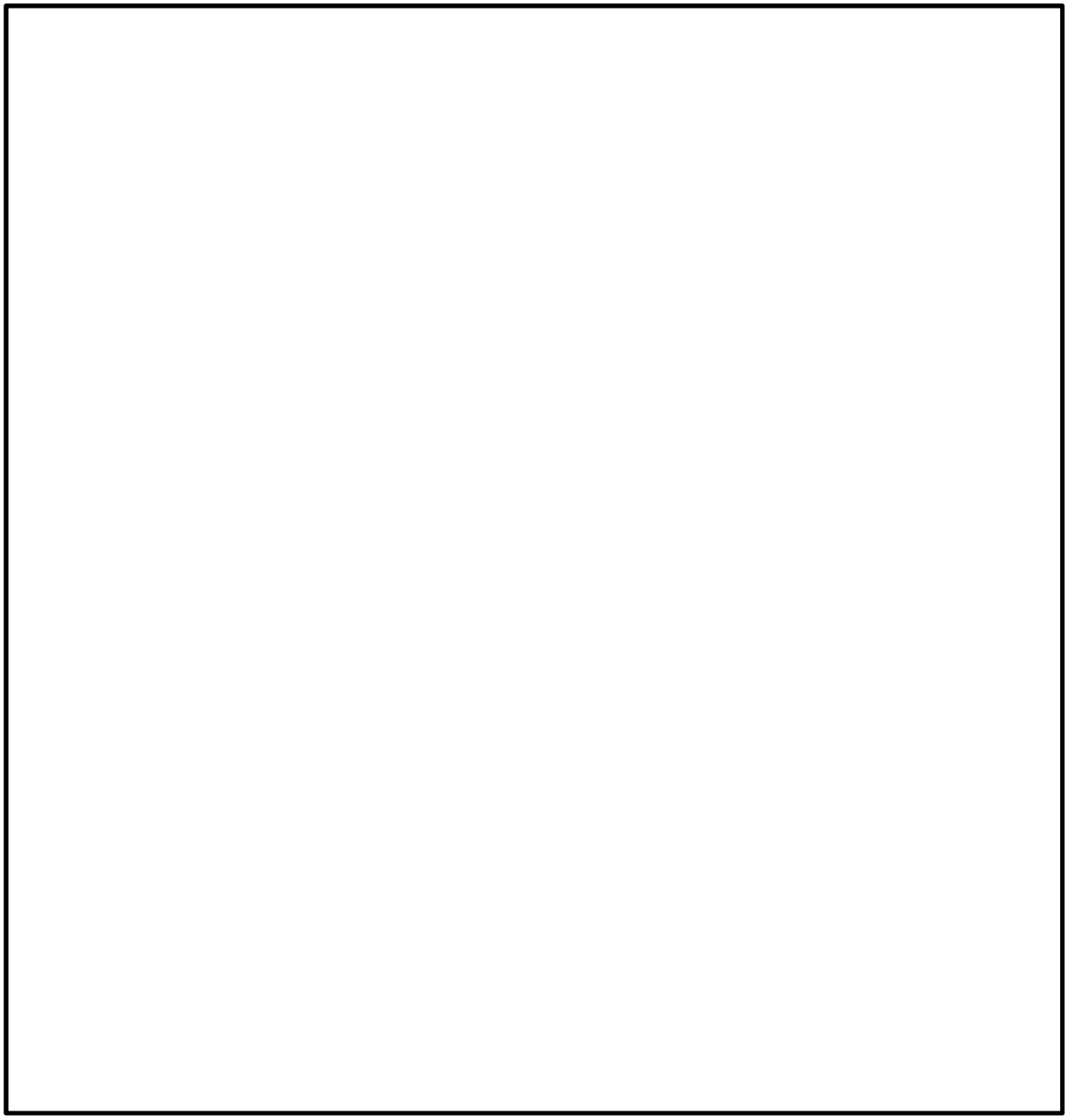


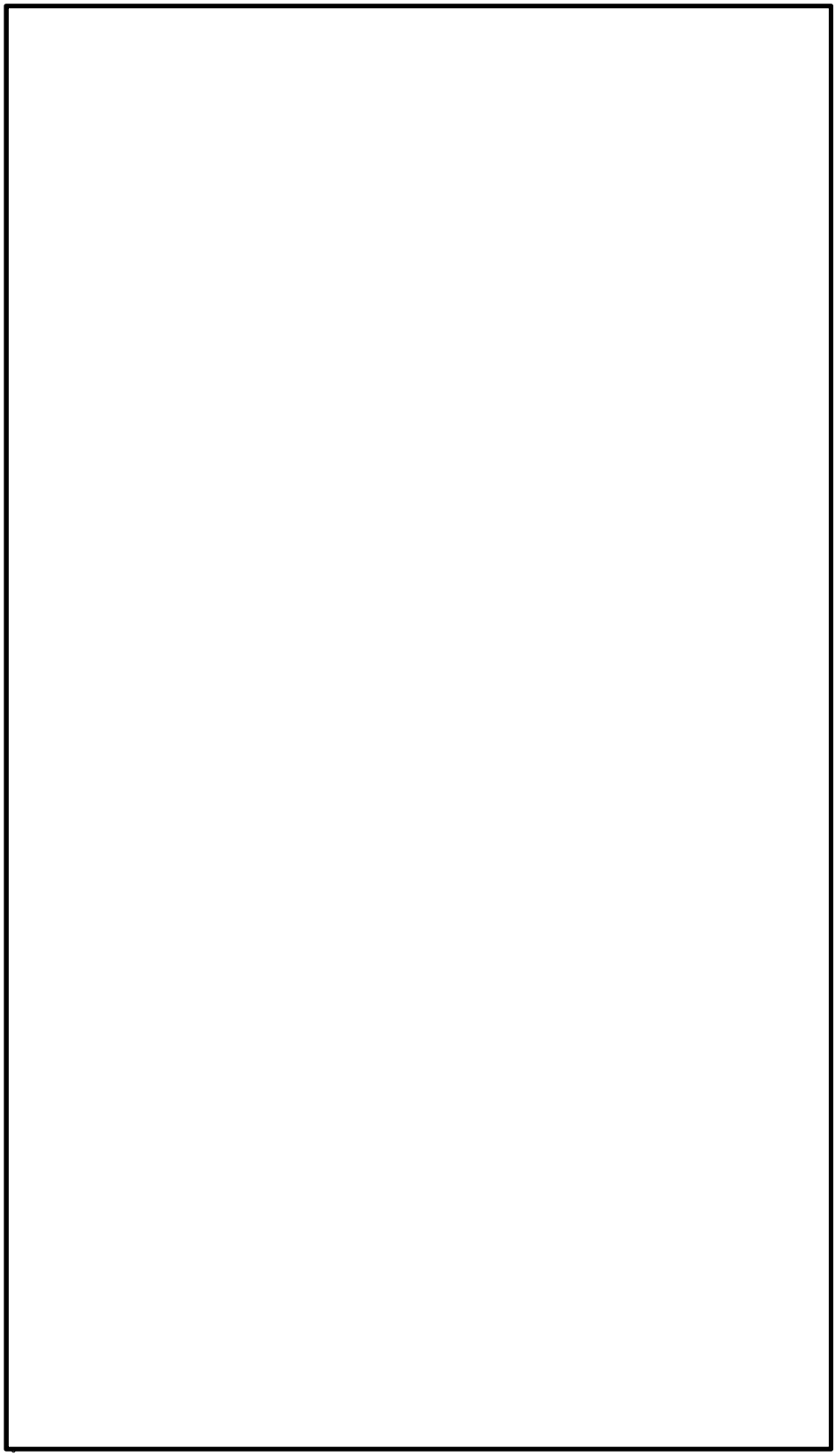


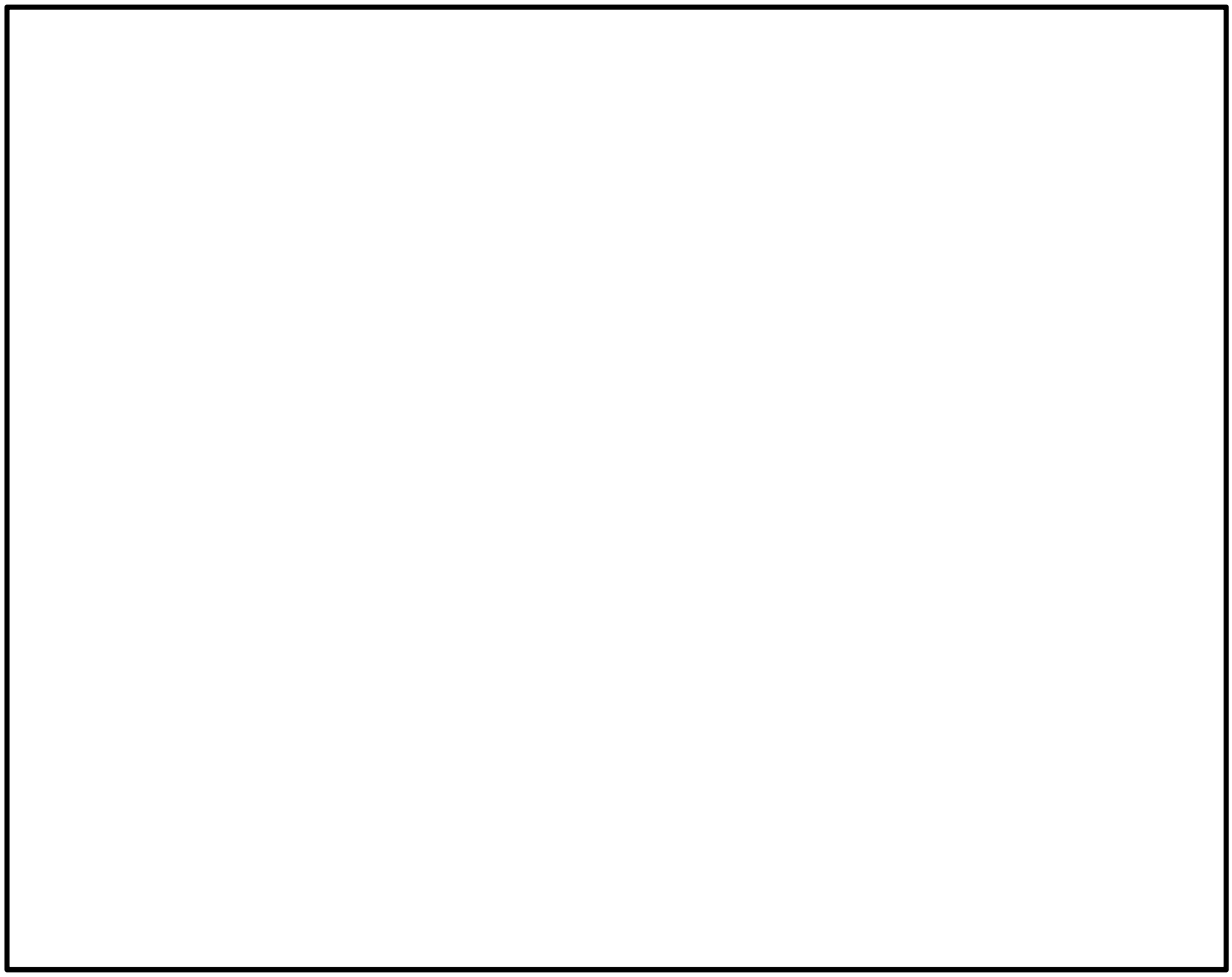


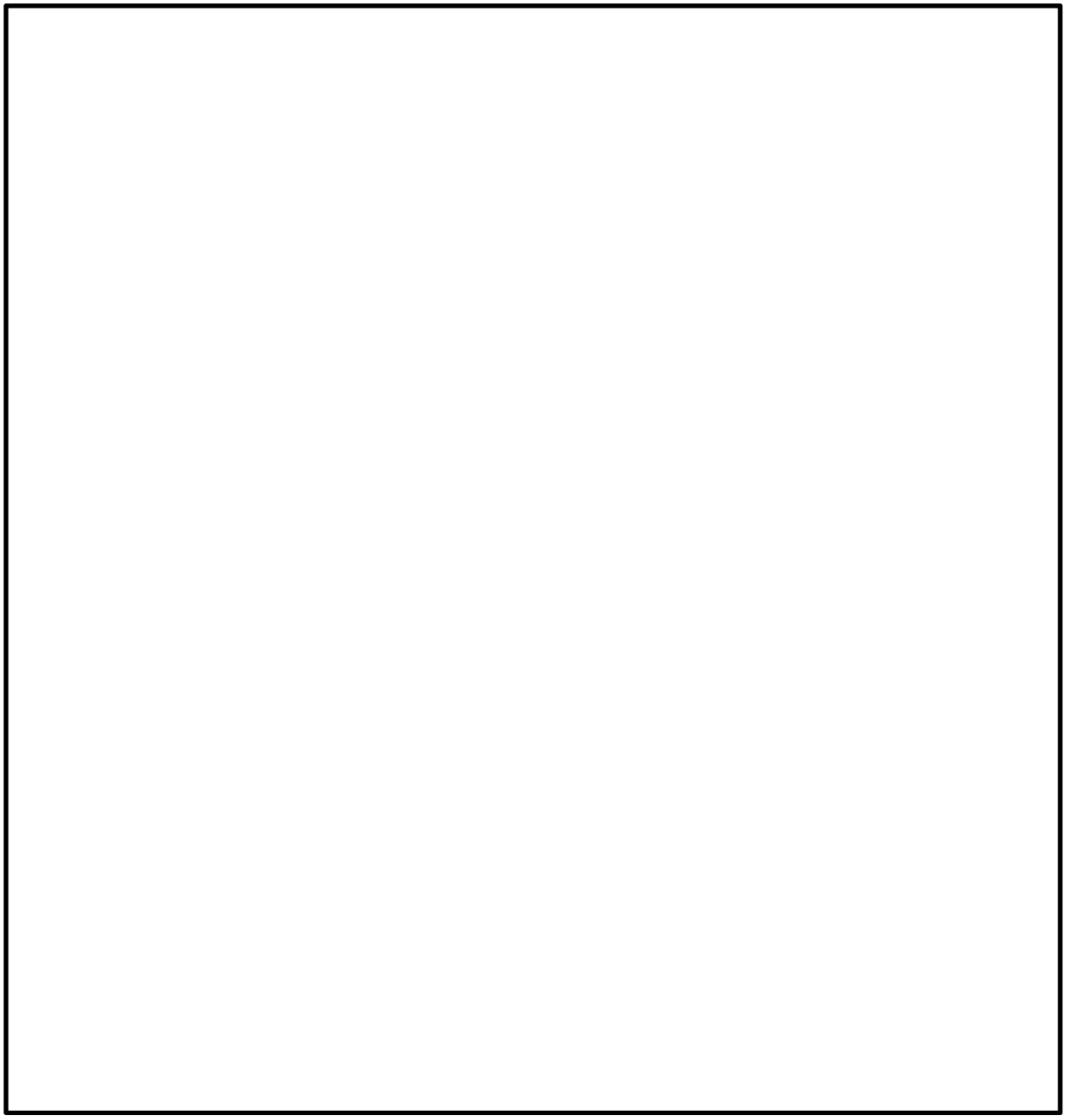


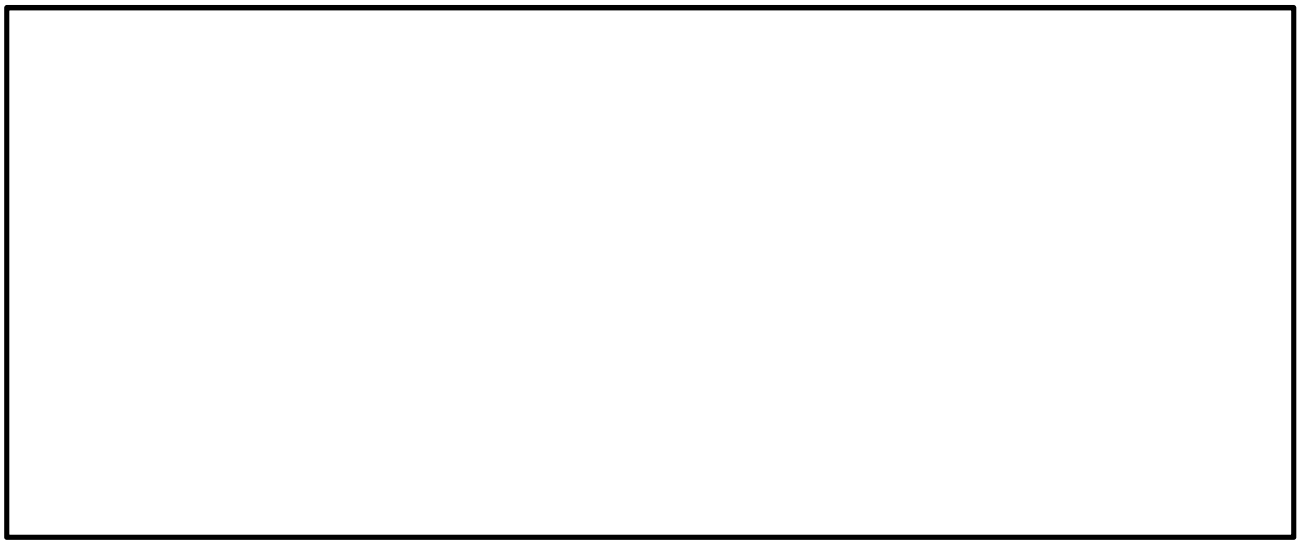


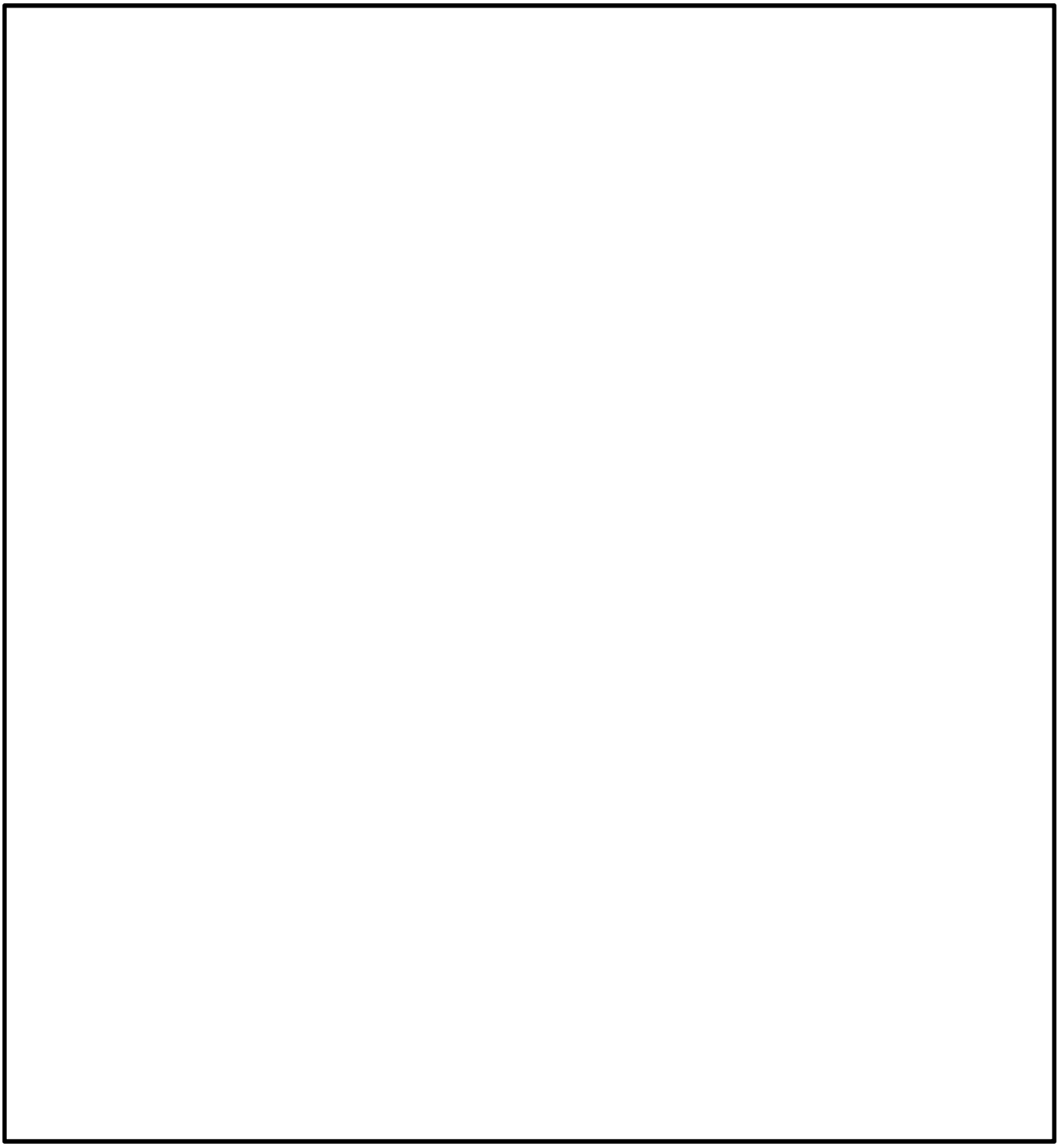


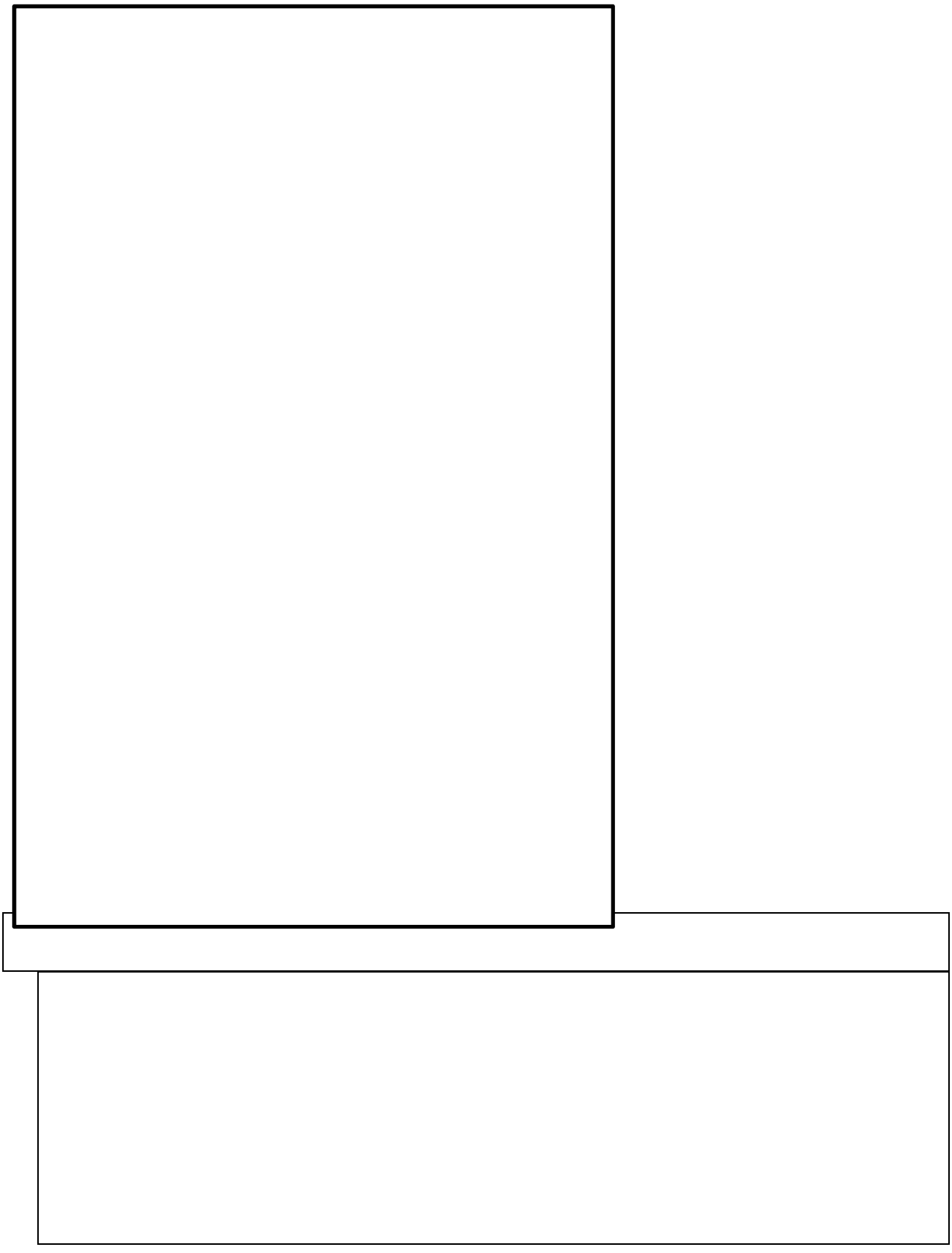












Appendix 4: Identification Kit – *S. cerevisiae* 3457

1. Introduction

The methods in this document are designed to allow one to determine whether an unknown yeast strain is the engineered [ ], or not. To aid in identification, one can use a combination of morphological and molecular characteristics of the production organism. The strain has the following key identifying characteristics,

2. Materials and Methods

2.1 Strains

2.2 Media

Synthetic minimal medium (SD) - used to culture the organism for phenotypic characterization.

	Per Liter
BD Difco Yeast nitrogen base	6.7 g
Glucose	20 g
Bacto agar	20 g

Combine Bacto agar with 900 ml of water. Autoclave. Dissolve Yeast nitrogen base, glucose, and urea in 100 ml water. Combine with Bacto agar while still hot. Pour into empty plates.

YPD Agar – used to grow strains for genomic DNA.

	Per 1 liter
Yeast extract	10 g
Bacto peptone	20 g
Glucose	20 g
Bacto agar	20 g

Bring to volume with MilliQ water. Autoclave.

2.3. Morphological and Physiological Characterization

Determination of Colony Morphology

1. Label sections of a YNB plate for each strain to be compared.

4. Inoculate the unknown fungus as above.
5. Incubate at 30 °C for 3 days.

3. Repeat with the unknown fungus.
4. Incubate at 30°C for 3 days.

Determination of Growth and Substrate Clearing

4. Repeat with the unknown fungus.
5. Incubate at 30°C for 4 days.

2.4 Molecular Biology Characterization

Chromosomal DNA Extraction and Purification.

Either amplify DNA using an extraction kit or follow the steps below

1. Grow strains overnight on YEPD agar.
2. Resuspend a loopful of cells in water, then gently pellet and discard supernatant.
3. Resuspend in 1 ml of Tris-buffered, pH7.5 1.2M sorbitol, with 50mM DTT. Incubate 30 minutes.
4. Add 10 ul of 12mg/ml Zymolase in 50% glycerol and incubate for two hours.
5. Pellet cells at 211 g (1.5k RPM on an Eppendorf 5424 24-place fixed angle rotor) for 10 minutes. Remove supernatant.
6. Lyse cells with 500 ul of SDS in Tris-EDTA buffer. Add 500 ul of 4.2M guanidinium hydrochloride, 0.9M potassium acetate, pH 4.8 buffer, and vortex thoroughly.
7. Centrifuge at 15800g (13k RPM on previously mentioned centrifuge) for 10 minutes.
8. Pipet supernatant into fresh tube and add an equal volume of isopropanol.
9. Centrifuge at 15800g for 10 minutes, discard supernatant.
10. Wash pellet in 100% ethanol, and air-dry.
11. Resuspend DNA pellet in 100ul of 10mM Tris-EDTA buffer, with 1ul of added RNAse.

PCR primers

Polymerase Chain Reaction (PCR) procedure

This protocol is to be used with purified chromosomal DNA. Each strain, the negative control strains and the unknown(s) should be tested for amplification.

For each sample to be tested, prepare three PCR reactions to test each set of primers. Mix the following components in a PCR tube:

Place the tube in a pre-programmed thermal cycler.
Program the automated thermal cycler according to manufacturers' instructions for the following program:

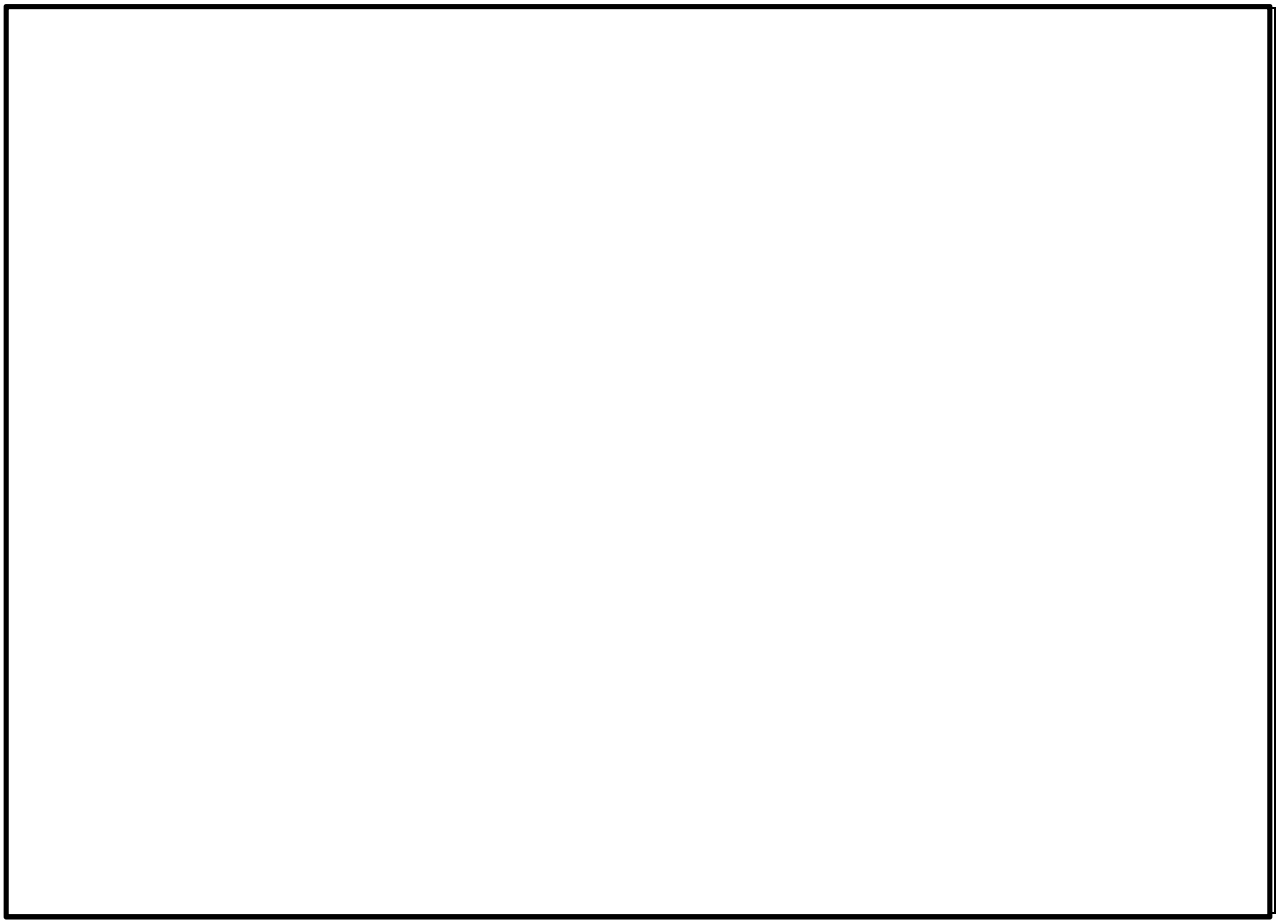
1. 95°C → 2 min
2. 94°C → 15 sec
3. 60°C → 20 sec
    a. Decrease by 0.5°C each cycle
4. 72°C → 90 sec
5. Go to step 2, repeat x10
6. 94°C → 15 sec
7. 51°C → 20 sec
8. 72°C → 90 sec
9. Go to step 2; repeat x18
10. 72°C → 3 min

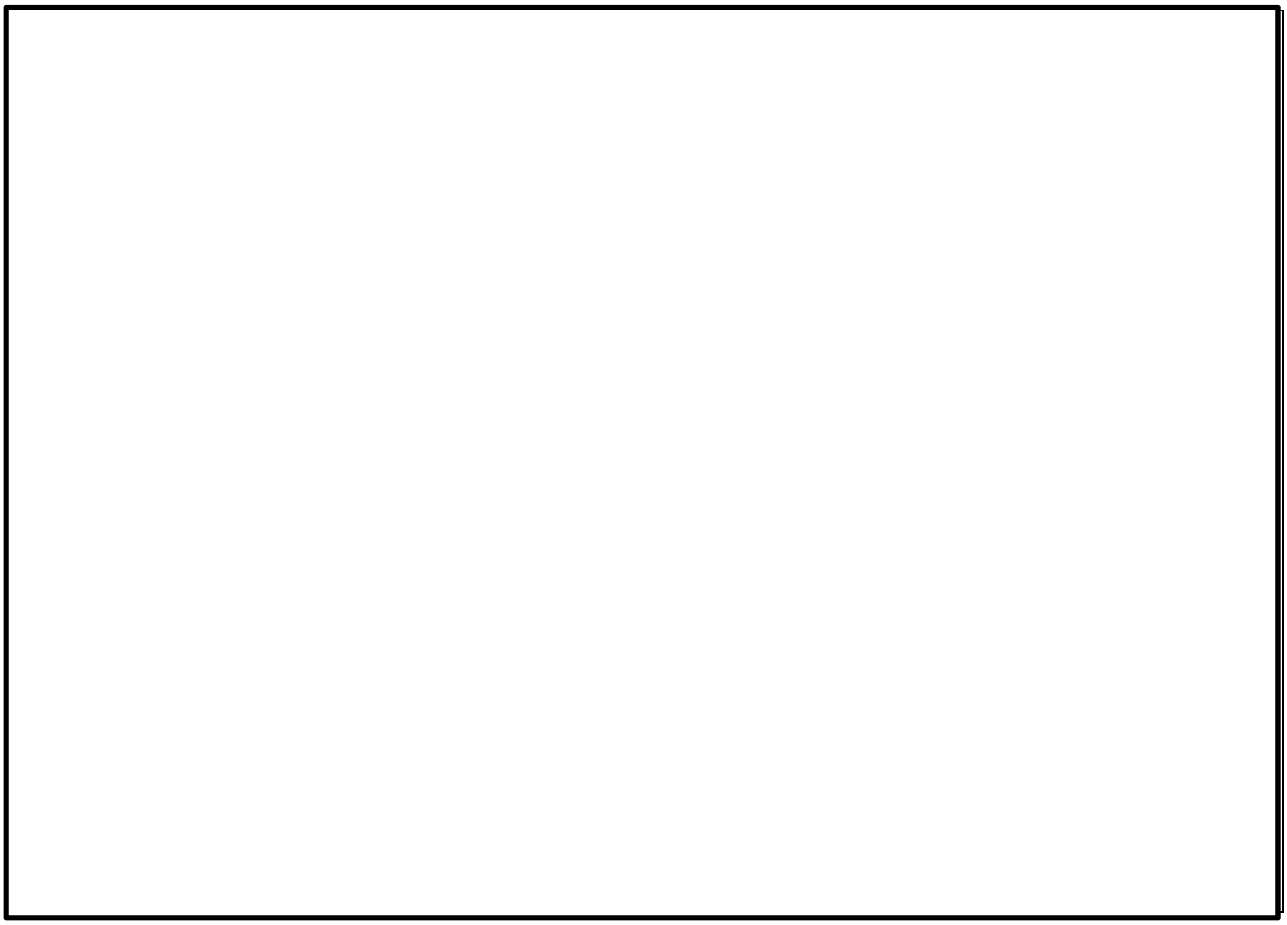
Electrophoresis of DNA samples

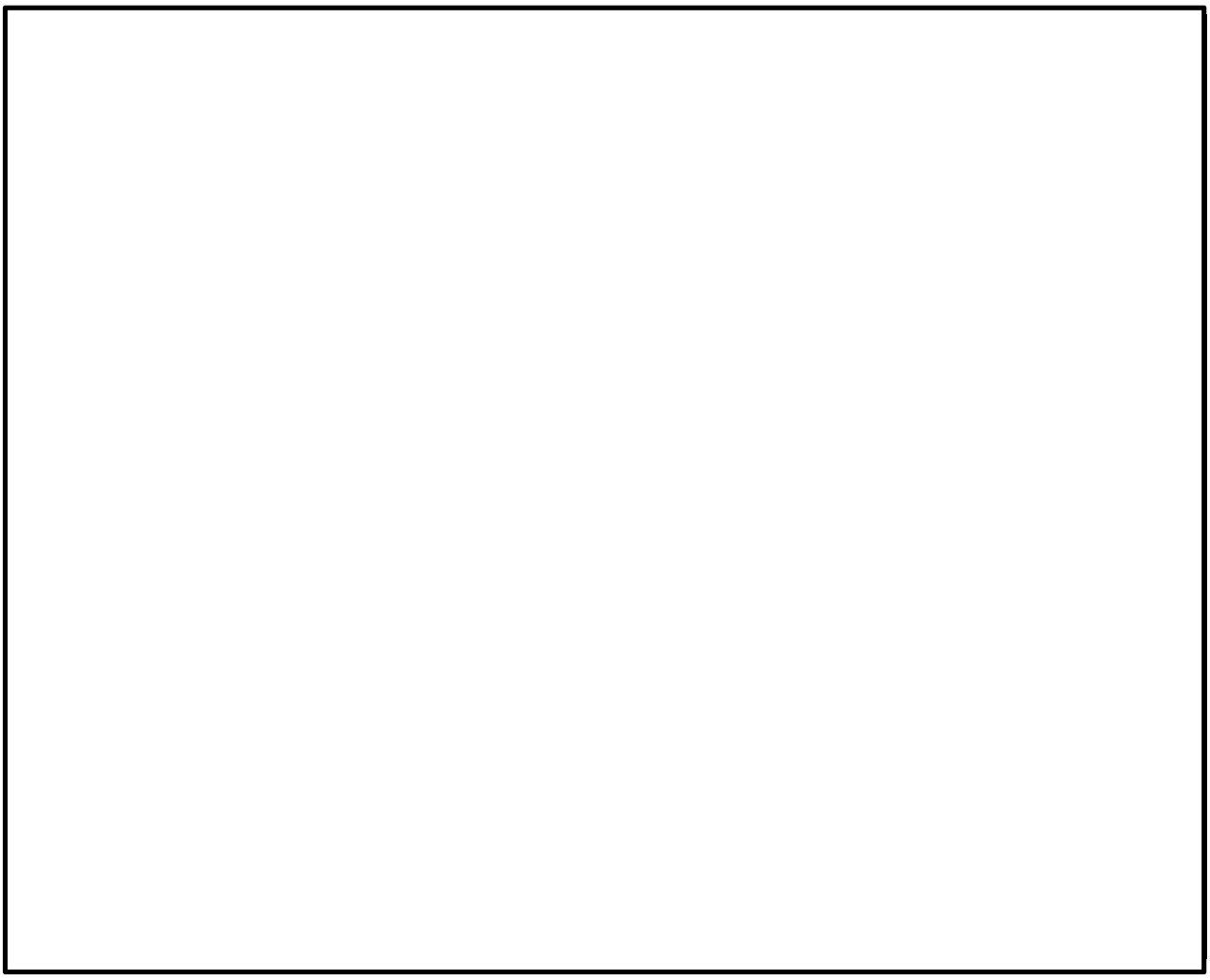
To analyze the DNA products, mix 10 µl of the PCR reaction with 10 µl of water. Load PCR mixture and Molecular Weight Marker (1 kb ladder) into the wells of a 1.2 % E-gel (Life Technologies), using a pipettor. With E-gel connected to the power supply, run for 30 minutes at 125V. Visualize using a UV lamp.

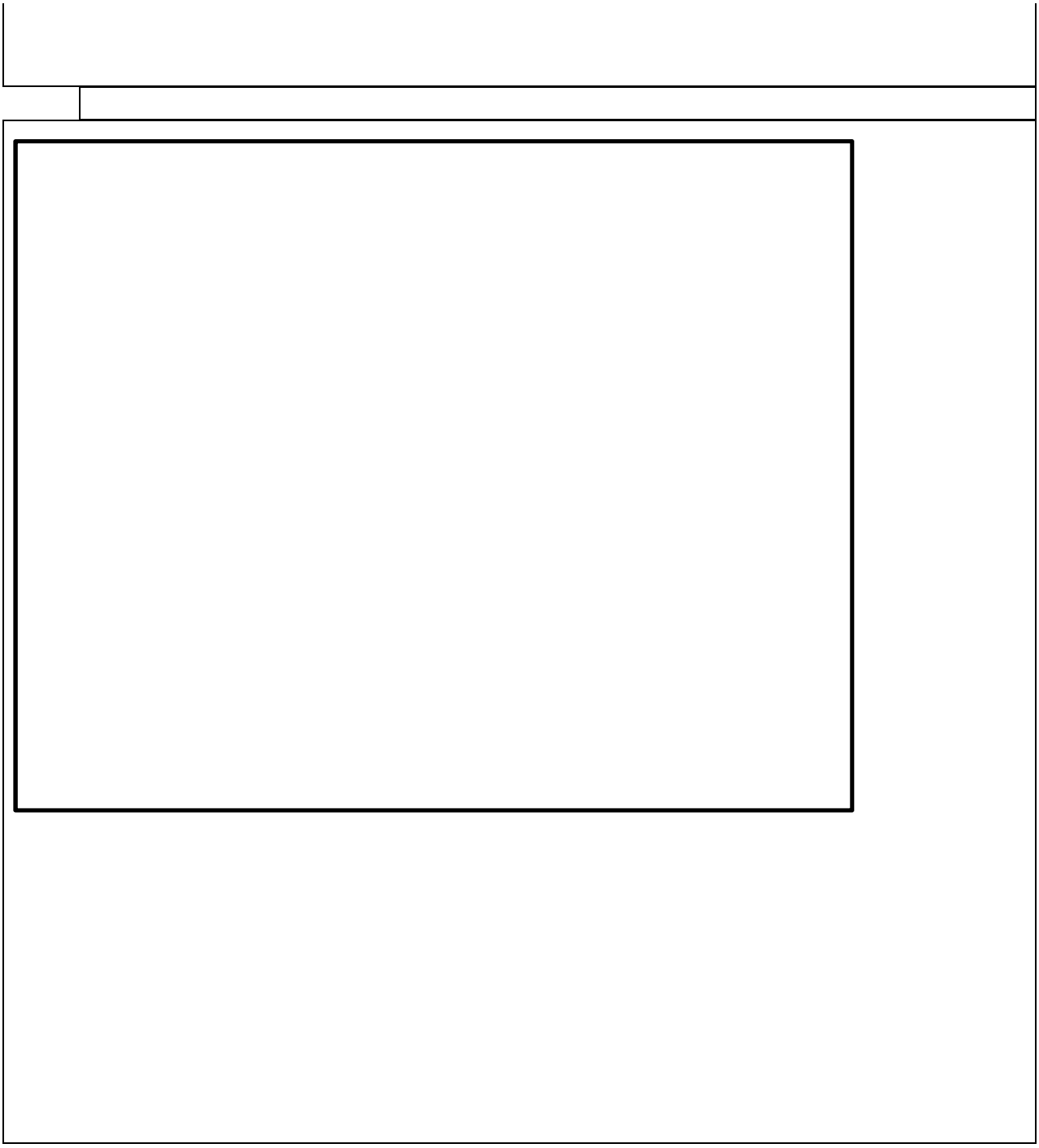
3

FIGURES









Appendix 5: Process Map for Production of Ethanol